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(56) Documents Cited
WO 2001/036587 A   US 4897354 A
J. Biol. Vol. 276 (22) 2001. Roeffen W F G et.al. pages 19807-19811
Blood Vol. 84 (5) 1994. Oeuvray C et.al. pages 1594-1602
Immunotechnology Vol. 2 (4) 1996. Dziegiel M et.al. page 299
J. Cellular Biol. (Suppl) 18D 1994. Sylvester D R et.al.

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(54) Abstract Title
Human antibodies against Plasmodium falciparum MSP-3 antigen

(57)   The construction of recombinant human antibodies against P. falciparum MSP-3 (merozoite surface protein), in particular MSP-3$_{194-257}$ antigen, is described. Iu is intended that the antibodies be used for the diagnosis and treatment of malaria. A method of screening a phage library with target molecules, such as MSP-3 antigen, bound to beads, preferably magnetic microspheres, is also described.

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|	RAM1-Fab	RAM2-Fab	RAM3-Fab	HibFab	PBS1%BSA
☐ anti-Fab	2,36	2,60	2,80	2,37	0,06
▦ MSP-3 aa 194-257	1,18	1,51	1,50	0,06	0,06
■ MSP-3 aa 22-257	1,04	1,22	0,13	0,06	0,06
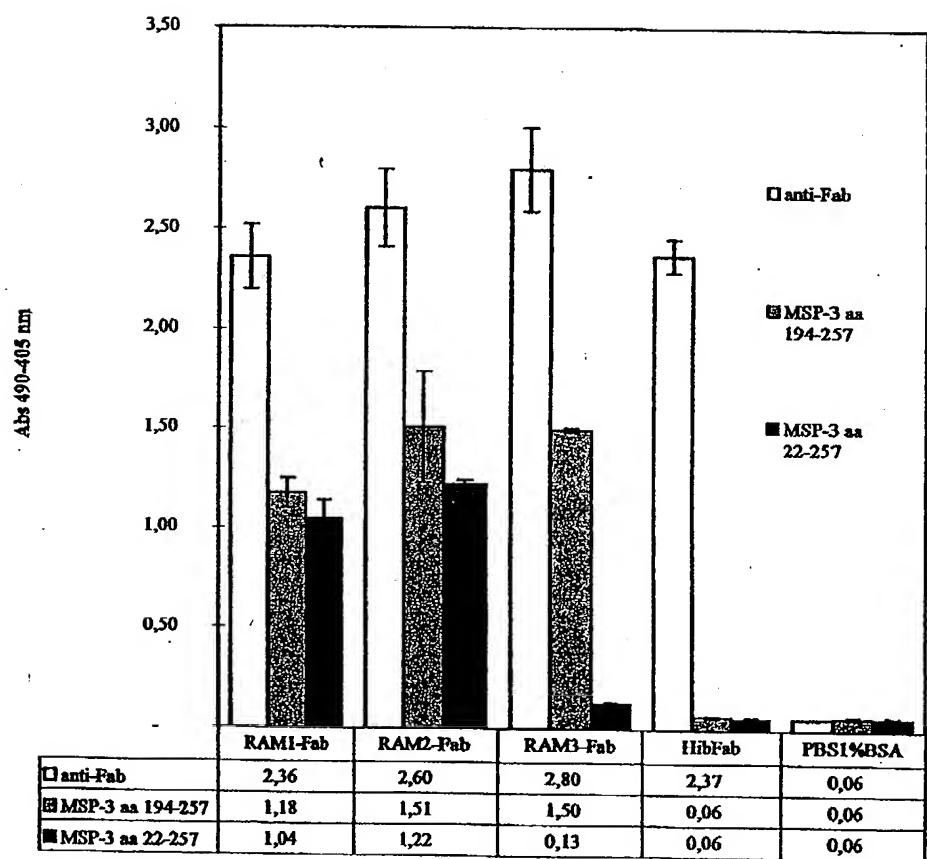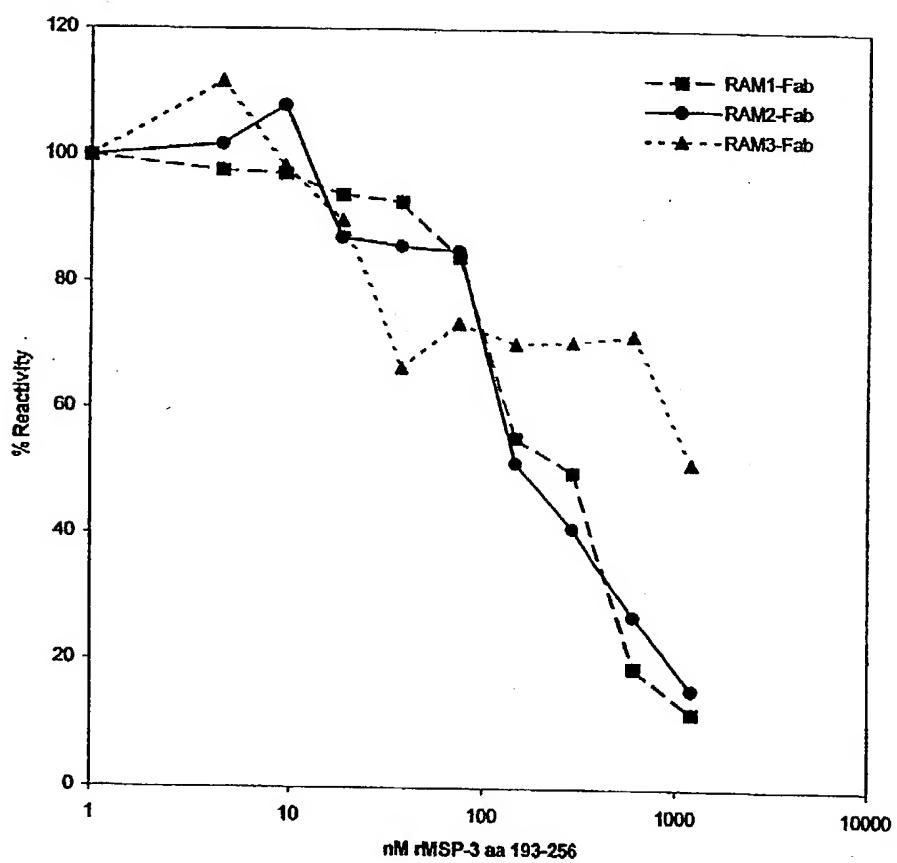
Figure 1

Figure 2

Figure 3

Figure 4

$$A2: 3.5\text{x}10^5 \longrightarrow A3: 17500 \longrightarrow A4: \quad 875$$

$$7\text{x}10^6 \longrightarrow B2: \quad 7\text{x}10^5 \longrightarrow B3: \quad 70000 \longrightarrow B4: \quad 7000$$

$$C2: 1.4\text{x}10^6 \longrightarrow C3: 2.8\text{x}10^5 \longrightarrow C4: 56\text{x}10^3$$

Figure 5

6/15

RAM1 VH.

QVQLVQSGGGVVQPGRSLRLSCAASGFTFS	SYAMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMSSLRAEDTAVYYCVK	GASS	WSTGLVTVSS	A
	CDR1		CDR2		CDR3		
DIVMQSPATLSASVGDRVTITC	RASQSISSWLA	WYQQKPGKAPKLLIY	KASSLES	GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC	QQYKSFPYT	FGQGTRLEIK	R

RAM1 Vk.

RAM2 VH.

QVQLQESGGGLVKPGGSLKLSCAASGFTFS	SYGMS	WVRQTPDKRLEWVA	TISSGGSYTYYPDSVKG	RFTISRDNAKNTLYLQMSSLKSEDTAMYYCAR	LYYGYRYFDY	WGQGTMVTVSS	A
	CDR1		CDR2		CDR3		
DIQLTQSPSSLSASIGDRVTITC	QASQDITNSLN	WYQQRPGQAPKLLIY	DAFILKT	GVPSRFSGRGSGTSFTIAINSFQAEDIGTYFC	QQSHRVPFT	FGGGTKVEIK	R

RAM2 Vk.

RAM3 VH.

| QVQLVQSGGGVVQPGRSLRLSCAASGFTFS	SYAMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDKAVYYCAR	DSGGIAARLGGYFDL	WGRGTTVTVSS	A
	CDR1		CDR2		CDR3		
DIRMTQSPSFLSASVGDRVTITC	RASQGISSYLA	WYQQKPGKAPKLLIY	AASTLQS	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC	QQGPT	FGGGTKVEIK	R

RAM3 Vk.

Figure 6
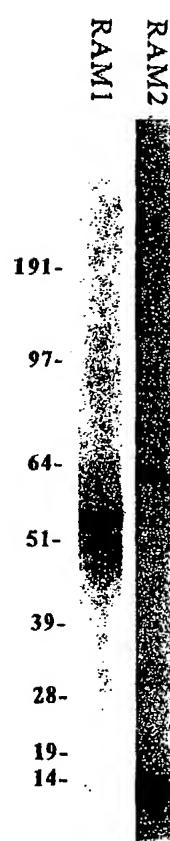
Figure 7
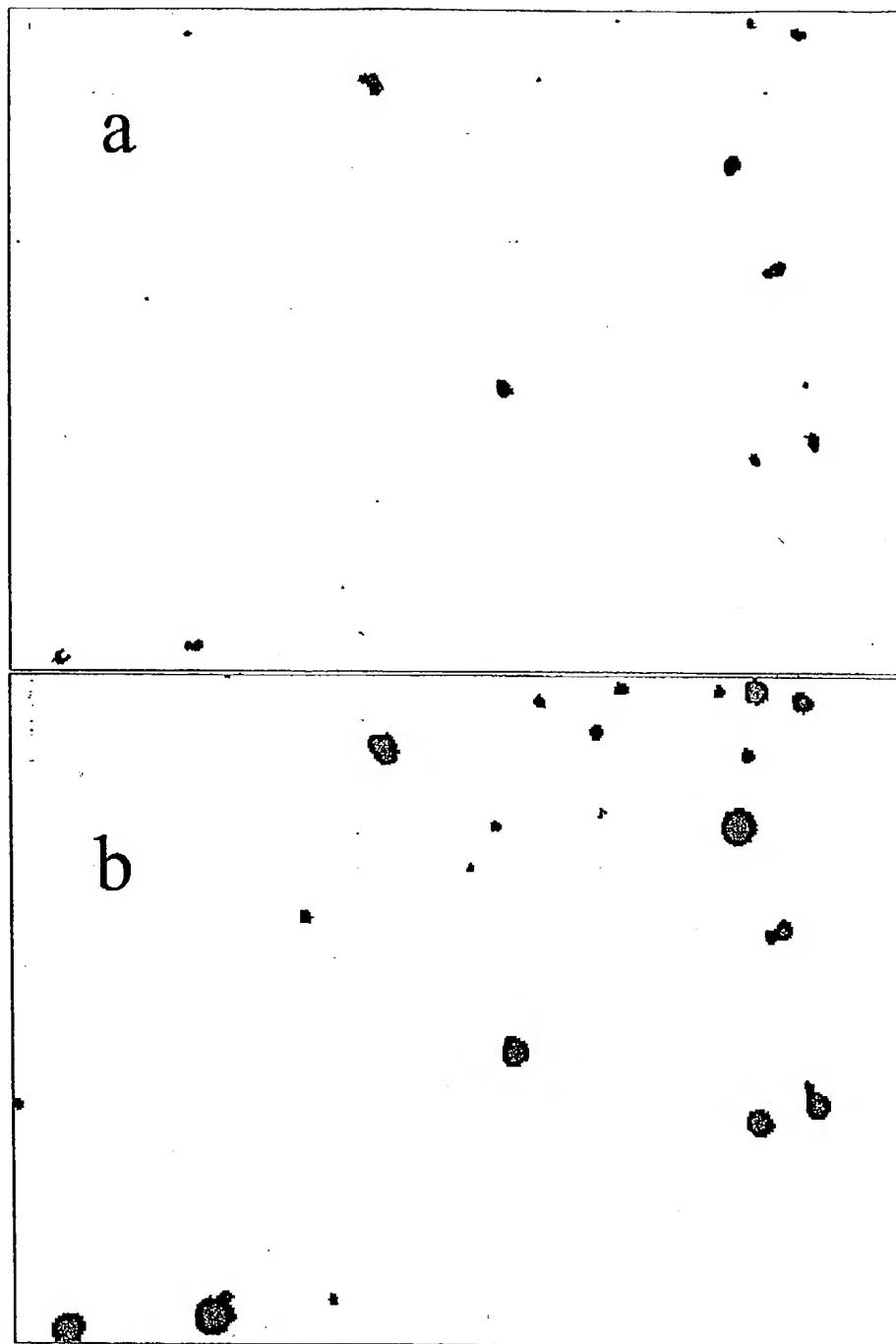
Figure 8
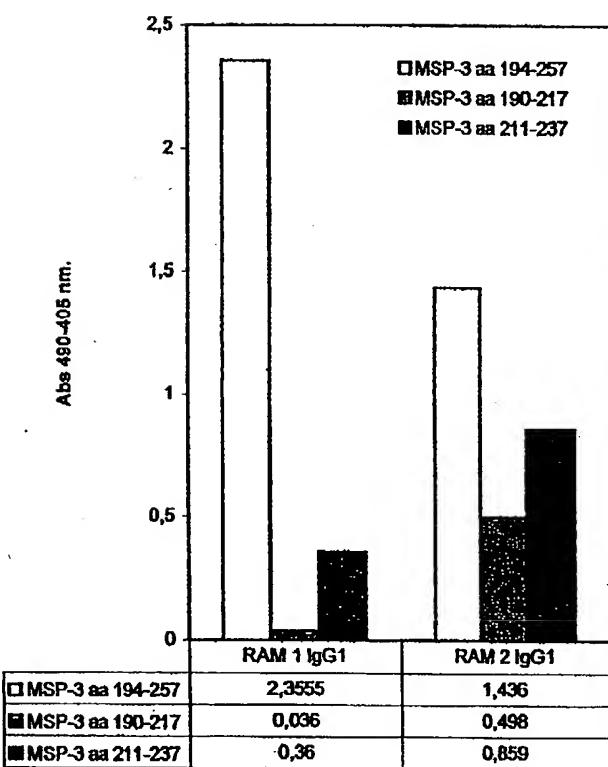
Figure 9
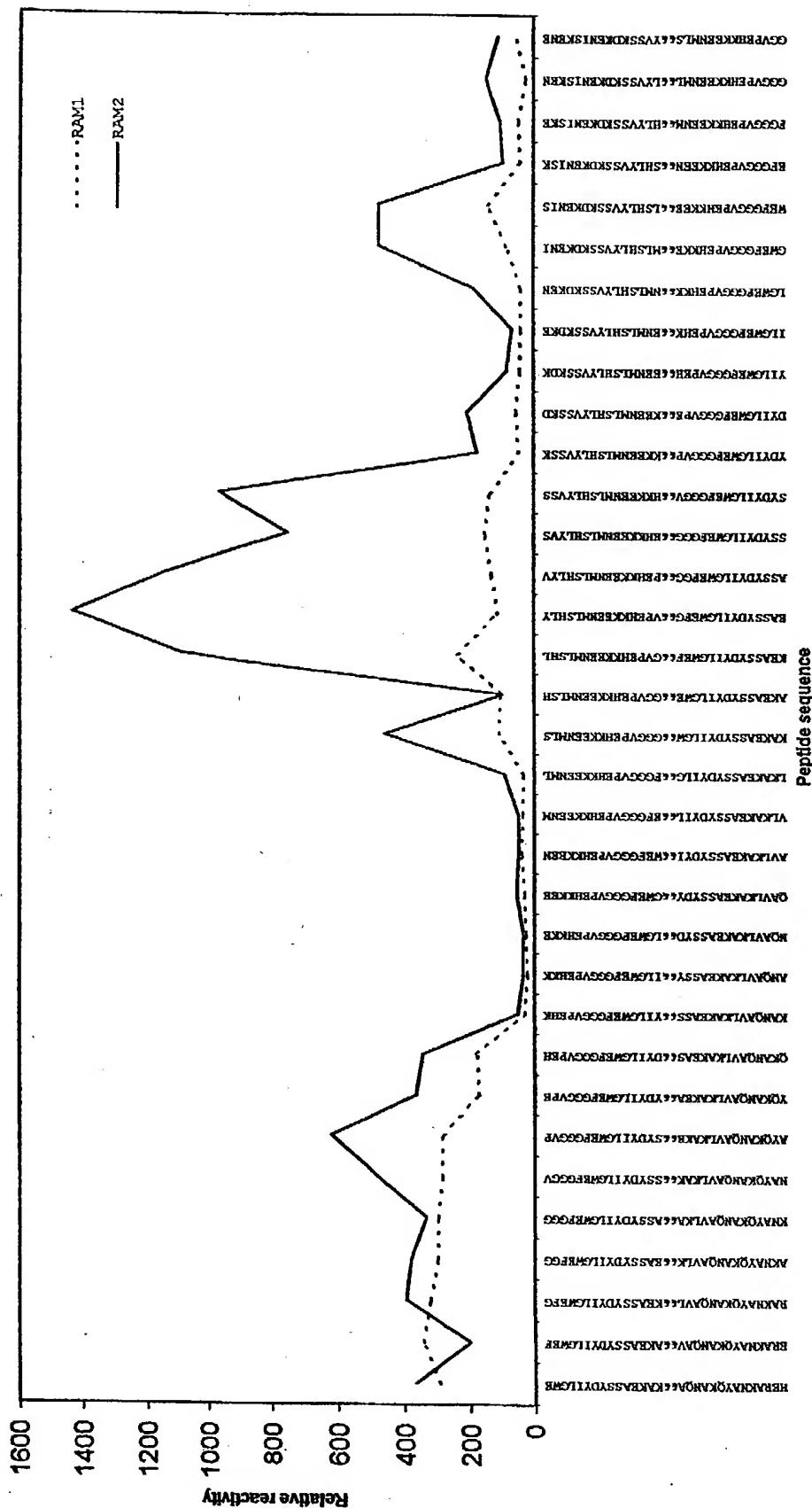
Figure 10
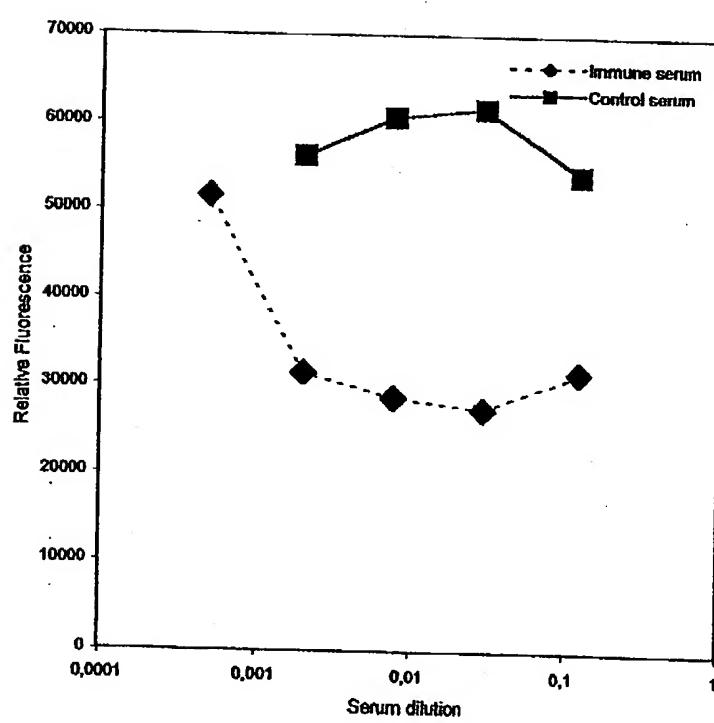
Figure 11
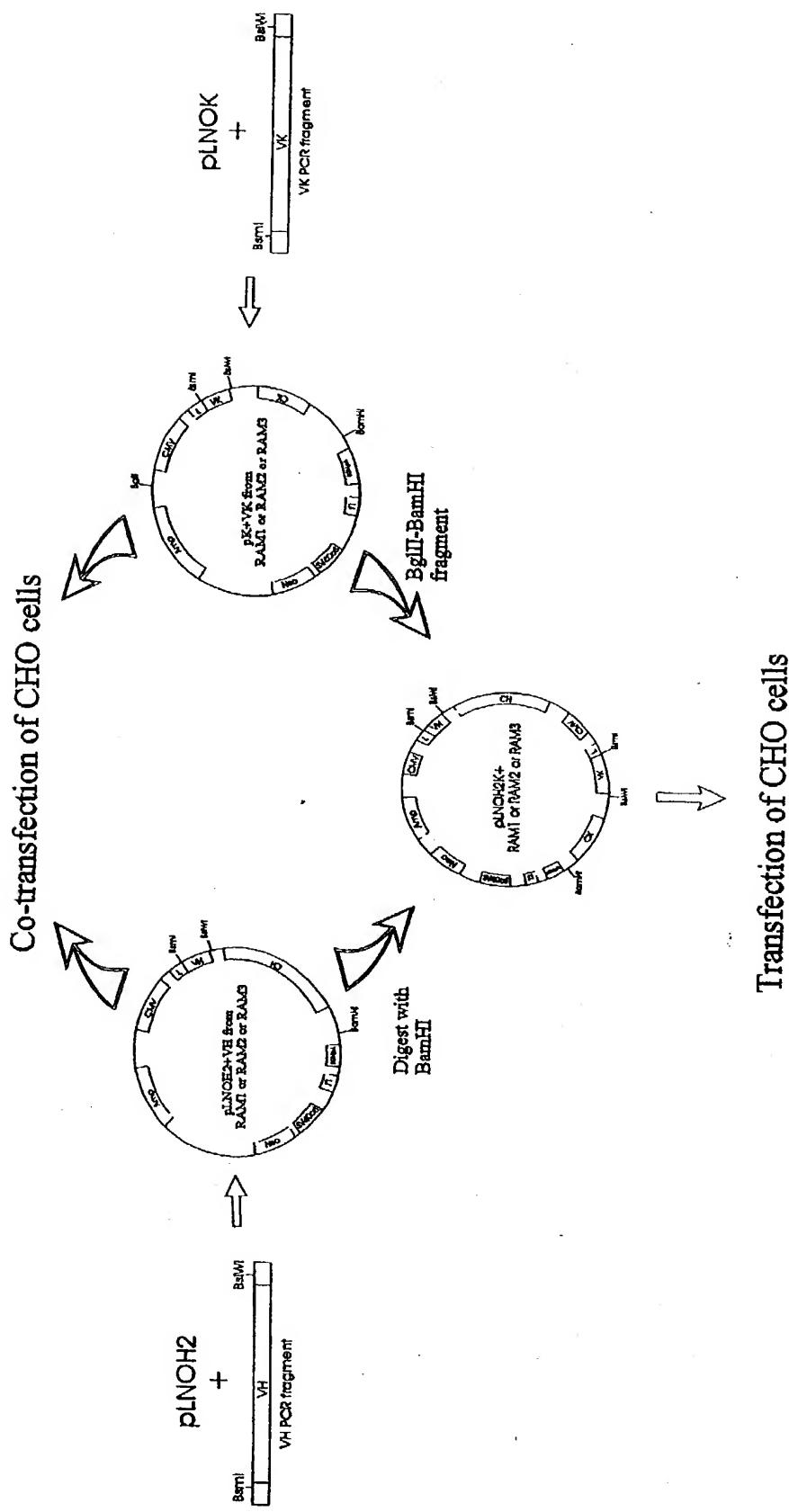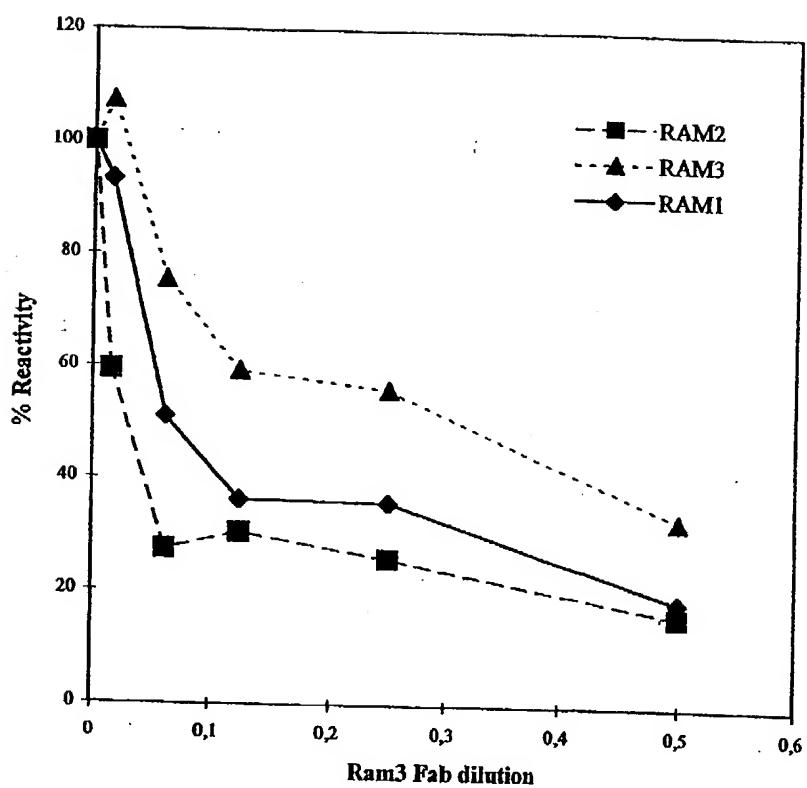
Figure 13
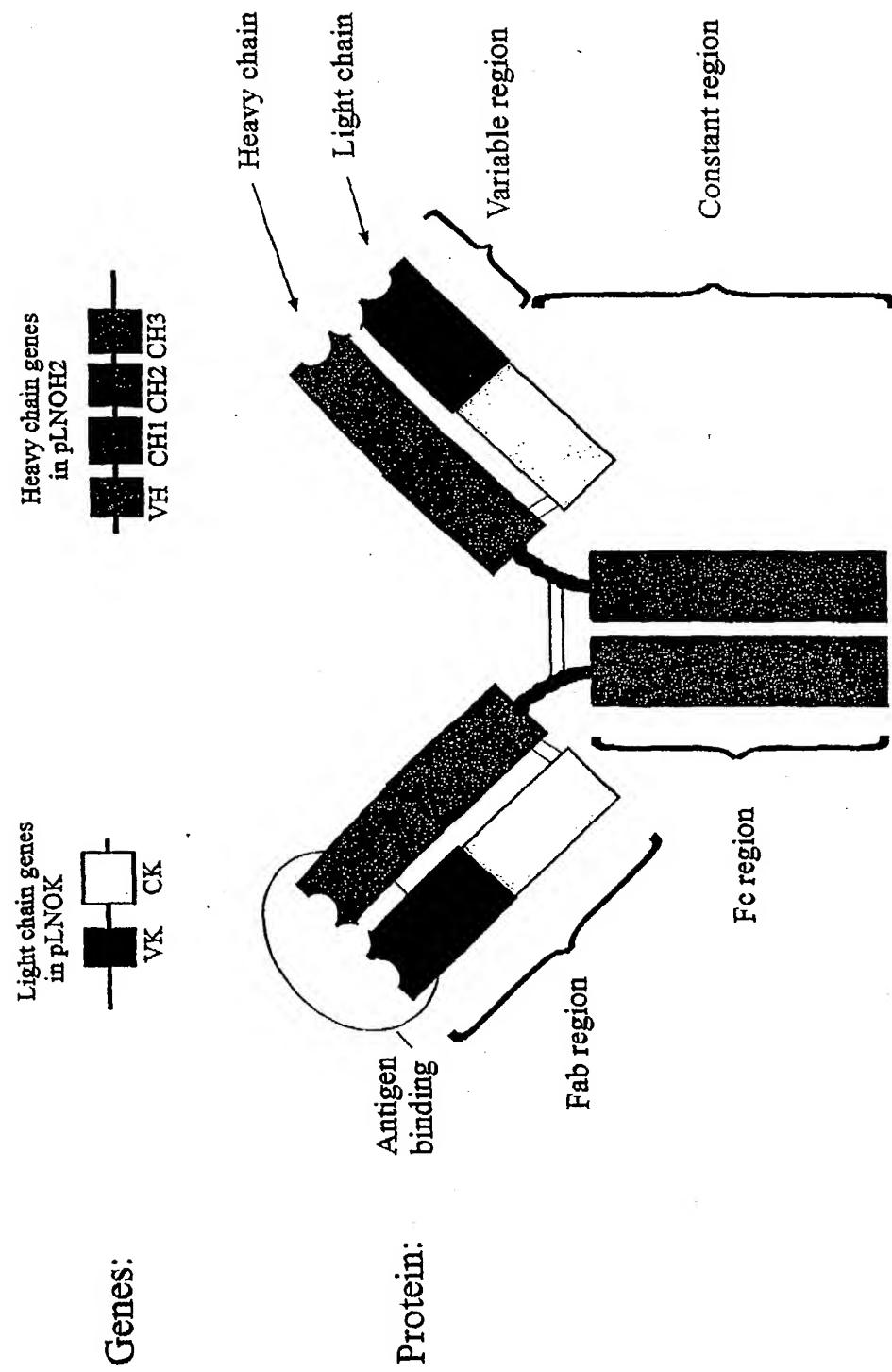
Figure 14

Table I
Results of screening of single clones from the three series after the fourth panning.

Series	Stringency Factor	Number of clones tested	Fab producers	Binders to MSP-3$_{193-256}$	Distribution Numbers in brackets
A	20	118	2	0	NA
B	10	132	9	8	RAM1 (7) + RAM3 (1)
C	5	126	12	9	RAM1 (7) + RAM2 (2)
Total		376	23	17	

Table 1

RECOMBINANT ANTI-PLASMODIUM FALCIPARUM ANTIBODIES

The present invention relates to recombinant antibodies against
the malaria parasite, e.g. *Plasmodium falciparum*, to methods of
producing such antibodies, and to uses and methods of use of
such antibodies.   In particular, the invention relates to
antibodies against a specific antigen; MSP-3.

PRIOR ART

Every year more than 2.5 million people, mostly children, die of
malaria.

The mortality and morbidity of this disease places a devastating
burden on the societies of the developing world.   The United
Nations has estimated that the loss of production due to malaria
in many African countries equals 5% of the gross national
product.

Resistance of the malaria parasite (*P. falciparum*) to the
existing anti-malarial drug chloroquine emerged in the sixties
and has been spreading since then.   In addition, the malaria
parasite has developed resistance to most other anti-malarial
drugs over the past decade.   This poses a major threat to public
health.   There is every reason to believe that the prevalence
and degree of anti-malarial drug resistance will continue to
increase.   Furthermore, many anti-malaria drugs have been
notorious for their toxic side effects, e.g. mefloquin.

It has been previously shown that anti-parasitic activity from
an immune donor can be transferred by IgG into non-immune
receivers (McGregor et al. 1963; McGregor 1964), and that such
antibodies do not act directly, but act indirectly through a
mechanism termed antibody-dependent cellular inhibition of
growth (ADCI, Bouharoun et al. 1990).   This   mechanism works
preferentially with IgG1 and IgG3 (Bouharoun-Tayoun et al. 1992;
Shi et al. 1999); (Aribot et al. 1996).

Using an ADCI assay, the merozoite surface antigen (MSP-3) was previously identified (Oeuvray et al. 1994). Affinity purified human polyclonal anti-MSP-3 antibodies have also been used in a mouse model (Badell et al. 2000).

There are a number of difficulties in using the antibodies described above, i.e. those purified from an infected host in the prevention or treatment of malaria. From a practical point of view it is highly unlikely that serum from infected individuals would ever be approved for medical use (for obvious safety reasons). Neither would sufficient amounts of antibodies be available.

In principle, recombinant human antibodies with appropriate specificity and activity were desirable. However, there have been difficulties in the art in developing these. For example, Seehaus et al. (1992) describes problems associated with the occurrence of deletion mutants in phage display protocols, which, despite being undesirable, tend to replicate faster than "intact" phages and so become over-represented.

DISCLOSURE OF THE INVENTION

The present inventors have, for the first time, developed recombinant human antibodies specific for the MSP-3 antigen. These antibodies are able to passively induce naturally occurring non-sterile malaria immunity (termed premunition) and exert their effect via the ADCI mechanism. Additionally, the recombinant antibodies may stimulate phagocytosis of infected erythrocytes and free parasites. Yet another mechanism of action of the antibodies may be direct interference with the function of the MSP-3 and/or the MSP-6 molecules by binding to the epitope.

Specifically, the inventors generated a phage display system based on the amplification of genes from the human peripheral blood leukocytes of 13 malaria immune individuals which encode variable regions (V) of antibody. These genes were isolated by the polymerase chain reaction (PCR).

Following amplification of the V region genes, these genes were cloned into a compatible phagemid vector, which directs expression of antibody fragments. The resultant phage display library was screened using a fragment of the merozoite surface protein, MSP-3; MSP-3$_{194-257}$ (Oeuvray et al. 1994).

Specifically, the inventors developed a new panning strategy to screen the library. In this method, biotinylated MSP-3$_{194-257}$ antigen was coupled to streptavidin coated polystyrene beads (Dynabeads), and incubated with the phage library.

The inventors thought that good binders would be present in very low frequency in the initial stock. To recover a high proportion of rare good binders, a high initial concentration of antigen was thought to be needed. However, in subsequent steps the good binders were assumed to be present with increasing frequency. So, the inventors reduced the amount of antigen used in each panning. This avoided the survival of mediocre binders.

The inventors strategy involved reduction of the number of antigen coated beads in subsequent pannings. In this way, both the amount of antigen and the area of matrix (the beads) are reduced concomitantly.

This concomitant reduction of the surface area of the matrix and the amount of antigen avoids a selective advantage of deletion mutants surviving by non-specific adherence to matrix.

The selection procedure resulted in isolation of three distinct clones designated RAM1, RAM2 and RAM3. RAM1 was predominant in the 376 phage-clones examined. Epitope mapping performed on these clones revealed the relationship between these clones.

The reactivity of the clones with native malaria antigen was demonstrated by immunoblotting, immunofluorescence and flow cytometry.

`The RAM1, RAM2 and RAM3 clones were produced as intact human IgG1 and intact IgG3 antibody in eukaryotic CHO cells. These antibodies have been purified to more than 99% purity.

Aspects of the invention

Accordingly, in a first aspect the present invention provides a recombinant human antibody comprising an antibody sequence specific for the MSP-3 antigen of *Plasmodium falciparum*.

An antibody is specific for a particular antigen if it binds that particular antigen in preference to other antigens. In particular, the antibody may not show any significant binding to molecules other than that particular antigen, and specificity may be defined by the difference in affinity between the target antigen and other non-target antigens. An antibody may also be specific for a particular epitope which may be carried by a number of antigens, in which case the antibody will be able to bind to the various antigens carrying that epitope. For example, specific binding may exist when the dissociation constant for a dimeric complex of antibody and antigen is 1 µM, preferably 100 nM and most preferably 1 nM or lower.

The recombinant human antibody of the invention is preferably specific for the MSP-3$_{194-257}$ antigen.

The recombinant human antibody of the invention may be specific for the epitope having the amino acid sequence ILGWEFGGGVP which corresponds to residues 220-230 of the MSP-3 antigen. This sequence is also conserved in other antigens such as the MSP-6 antigen of *Plasmodium falciparum* (Trucco et al. 2001).

The recombinant human antibody may comprise a CDR sequence selected from: CDR1 of RAM1 VH; CDR2 of RAM1 VH; CDR3 of RAM1 VH; CDR1 of RAM1 VK; CDR2 of RAM1 VK; CDR3 of RAM1 VK; CDR1 of RAM2 VH; CDR2 of RAM2 VH; CDR3 of RAM2 VH; CDR1 of RAM2 VK; CDR2 of RAM2 VK; CDR3 of RAM2 VK; CDR1 of RAM3 VH; CDR2 of RAM3 VH; CDR3 of RAM3 VH; CDR1 of RAM3 VK; CDR2 of RAM3 VK; or CDR3 of

RAM3 VK, having the sequences shown in Figure 6 (the CDRs are marked and are underlined on Figure 6).

Preferably, the antibody comprises the CDR3 from a heavy chain, that is a CDR selected from: CDR3 of RAM1 VH; CDR3 of RAM2 VH; or CDR3 of RAM3 VH, having the sequences as shown in Figure 6.

More preferably, the antibody comprises two or more CDRs shown in Figure 6, i.e. two or more CDRs selected from CDR1 of RAM1 VH; CDR2 of RAM1 VH; CDR3 of RAM1 VH; CDR1 of RAM1 VK; CDR2 of RAM1 VK; CDR3 of RAM1 VK; CDR1 of RAM2 VH; CDR2 of RAM2 VH; CDR3 of RAM2 VH; CDR1 of RAM2 VK; CDR2 of RAM2 VK; CDR3 of RAM2 VK; CDR1 of RAM3 VH; CDR2 of RAM3 VH; CDR3 of RAM3 VH; CDR1 of RAM3 VK; CDR2 of RAM3 VK; or CDR3 of RAM3 VK having the sequence shown in Figure 6.

The antibody may comprise any one of CDR1, CDR2, and CDR3 of a heavy chain as shown in Figure 6 and any one CDR1, CDR2 and CDR3 of a light chain as shown in Figure 6. For example the antibody may comprise CDR3 of a heavy chain and CDR1 of a light chain. Or, the antibody may comprise two or more CDRs from a heavy chain, or two or more CDRs from a light chain as shown on Figure 6.

Most preferred is that the antibody comprises CDR1, CDR2 and CDR3 of a light chain as shown in Figure 6, and CDR1, CDR2 and CDR3 of a heavy chain as shown in Figure 6.

It is preferred that, where the antibody comprise two or more CDRs, the two or more CDRs are from the same RAM clone, that is two or more CDRs from RAM1; two or more CDRs from RAM2 or two or more CDRs from RAM3.

It is most preferred that the antibody comprises the CDR1, CDR2, CDR3 of the light chain and CDR1, CDR2 and CDR3 of the heavy chain, wherein all six CDRs are from the same RAM clone, e.g. all six CDRs are from RAM1.

Although it is preferred that within a single antibody the CDRs
are from a single RAM clone, e.g. RAM1, RAM2 or RAM3, any
combination is possible, that is where the antibody comprises
two or more CDRs as disclosed herein, a first CDR may be from
any one of RAM1; RAM2 and RAM3 and the second CDR may be from
any of RAM1; RAM2 or RAM3. To produce combinations in which
CDRs derive from different RAM clones, CDR shuffling (Jirholt P,
Ohlin M, Borrebaeck CA, Soderlind E. (1998). Exploiting
sequence space: shuffling in vivo formed complementarity
determining regions into a master framework. Gene 1998 Jul 30;
215(2):417-6) or DNA shuffling may be used (Crameri et al.,
1996).

Preferably, the recombinant human antibody may comprise a V_H
domain (Variable domain of the heavy chain) selected from RAM1
VH; RAM2 VH or RAM3 VH having the amino acid sequence shown in
Figure 6 (the VH amino acid sequences are shown as the top line
for each RAM clone in Figure 6).

Preferably, the recombinant human antibody has a V_L domain
(Variable domain of the light chain) selected from RAM1 VK; RAM2
VK; or RAM3 VK having the amino acid sequence of RAM1, RAM2 or
RAM3 shown in Figure 6 (the VK amino acid sequences are shown as
the bottom line for each RAM clone in Figure 6). Any light
chain may be combined with any heavy chain, e.g., by light and
heavy chain shuffling (Marks et al. 1992).

However, it is preferred that the recombinant human antibody has
the V_L domain of RAM 1 VK and the VH domain of RAM1 VH; or the VL
domain of RAM2 VK and the VH domain of RAM2 VH; or the VL domain
of RAM3 VK and the VH domain of RAM3 VK, as shown in Figure 6.

The variable heavy chain regions discussed above may be combined
with any suitable constant region, including the constant region
of gamma 1, gamma 2, gamma 3, gamma 4, my, alfa 1, alfa 2, delta
or epsilon isotypes as well as any artificial constant region.

For each isotype, any subclass and any allotype may be used.
Alternatively, other molecules capable of eliciting the desired

effector functions or anti-parasitic effects may be coupled to
the antigen-binding region of the antibodies, e.g. enzymes with
anti-parasitic effects. For example, the use of such antibodies
may confer target selectivity to an otherwise toxic drug or
substance.

It is preferred that the recombinant human antibody comprises a
constant region of the gamma 1, gamma 2, gamma 3, or gamma 4
isotypes, to form an IgG molecule.

More preferably, the recombinant human antibody comprises a
constant region of the gamma 1 or the gamma 3 isotypes, to form
an IgG1 or IgG3 isotype.

The recombinant human antibodies of the invention may be
produced by expression from a suitable nucleic acid molecule.

In addition to an antibody sequence, the antibody may comprise
other amino acids, e.g. to impart to the molecule another
functional characteristic in addition to ability to bind
antigen. For example, the specific binding member may comprise
a label, or enzyme and so on (as discussed in more detail
below).

In addition to the complete antibody, fragments of the antibody
may also have the ability to bind the appropriate antigen (such
as MSP-3), and are therefore also encompassed by the invention.
For example, it has been shown that fragments of a whole
antibody can perform the function of binding antigens. Examples
of binding fragments are (i) the Fab fragment consisting of VL,
VH, CL and CH1 domains; (ii) the Fd fragment consisting of the
VH and CH1 domains; (iii) the Fv fragment consisting of the VL
and VH domains of a single antibody; (iv) the dAb fragment
(Ward, E.S. et al. Nature 341, 544-546 (1989)) which consists of
a VH domain; (v) isolated CDR regions; (vi) F(ab')2 fragments, a
bivalent fragment comprising two linked Fab fragments (vii)
single chain Fv molecules (scFv), wherein a VH domain and a VL
domain are linked by a peptide linker which allows the two
domains to associate to form an antigen binding site (Bird et

al, Science, 242, 423-426, 1988; Huston et al, PNAS USA, 85, 5879-5883, 1988); (viii) bispecific single chain Fv dimers (PCT/US92/09965) and (ix) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Holliger et al, Proc. Natl. Acad. Sci. USA 90 6444-6448, 1993). Fv, scFv or diabody molecules may be stabilised by the incorporation of disulphide bridges linking the VH and VL domains (Y. Reiter et al, Nature Biotech, 14, 1239-1245, 1996). Minibodies comprising a scFv joined to a CH3 domain may also be made (S. Hu et al, Cancer Res., 56, 3055-3061, 1996).

Accordingly, in a further aspect the present invention provides an isolated nucleic acid molecule comprising a nucleic acid sequence encoding a recombinant human antibody of the first aspect.

Such a nucleic acic molecule may be in the form of a recombinant and preferably replicable vector.

Such a 'vector' may be any plasmid, cosmid, or phage in double or single stranded linear or circular form which may or may not be self transmissible or mobilizable, and which can transform prokaryotic or eukaryotic host either by integration into the cellular genome or exist extrachromosomally (e.g. autonomous replicating plasmid with an origin of replication). Any suitable host may be used, including bacteria, e.g. archaebacteria, plants, plant cell, fungi.

Generally speaking, those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression. For further details see, for example, *Molecular Cloning: a Laboratory Manual:* 2nd edition, Sambrook *et al,* 1989, Cold Spring Harbor Laboratory Press (or later editions of this work)and *Current Protocols in Molecular Biology,* Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992, which are incorporated herein by reference.

Preferred vectors include the plasmids pLNOH2 or pLNOK which are described in Norderhaug et al. 1997 (and discussed in more

detail later). Other suitable vectors for expresssion of antibodies are described in Sanna et al. 1999; Persic et al. 1997; Walls et al. 1993.

5   Plasmids pLNOH2 and pLNOK may be expressed by any suitable cell type, e.g. mammalian, yeast, insect or plant cells, especially mammalian cells such as BHK, CHO, or COS cells, using methods which are standard in the art. CHO or BHK cells are most preferred.

10

It is preferred that the antibody is secreted to the media by the cell from which it is expressed.

Further aspects of the invention relate to: a method of
15   expressing in a host cell an antibody as described herein from a nucleic acid molecule described herein; a host cell capable of expressing an antibody as described herein in appropriate culture conditions for producing said antibody; a method of producing an antibody comprising culturing such a host cell
20   under appropriate conditions, which method may further comprise isolating said antibody from the cell culture, and which method may further comprise admixing the isolated antibody with a suitable further component (which may, for example, be another antibody or an excipient or carrier).

25

Mammalian cells may be transfected by any suitable technique such as lipofection. One suitable method is described in the Examples. Alternatively, standard calcium phosphate transfection or electroporation may be used, which is well
30   understood by the skilled person.

The recombinant antibodies produced from these expression systems and nucleic acid molecules of the invention are preferably provided in a substantially pure or homogeneous form.
35   Recombinant antibodies may be purified by any suitable method such as ammonium sulphate precipitation, preferably followed by purification using a DEAE Sepaharose column and an ABx column (Baker Bond). This may optionally be followed by a gel filtration step, e.g. using Superdex200.

In a further aspect of the invention, there is provided a method for screening a phage display library, comprising the following steps:

    (i)   attaching a target molecule (such as an antigen) to a bead using a suitable linker;

    (ii)   admixing a first amount of such beads with attached target molecule with a first population of phage from a phage display library (which library expresses sequences (such as antibody sequences) which may bind to the target molecule;

    (iii)   selecting from the first population of phage a second population of phage, wherein the second population of phage is enriched in phage which bind to the target molecule;

    (iv)   admixing said second population of phage with a second amount of beads, wherein said second amount of beads is smaller than said first amount;

    (v)   selecting from said second population of phage a third population of phage, wherein said third population of phage enriched in phage which bind to the target molecule.

Steps (iv) and (v) may be repeated to produce fourth and fifth populations.

Using this method, the amount of target molecule and area of binding matrix (total bead surface area) are reduced concomitantly.

It is preferred that the target molecule is an antigen and the phage display library contains antibody sequences.

A suitable linker for attachment of the target molecule to the bead is an avidin-biotin linker.

A suitable reduction between the first and second amounts of beads is a reduction by a factor of 10. For example, where the

first amount of beads is 7x 10^5 the second amount of beads may be 70000. If steps (iv) to (v) are repeated then a third amount of beads may be around 7000.

Other reductions in the amount of beads are also possible and can be varied by the skilled person. The reductions do not need to be the same throughout the method. For example, the reduction between first and second amounts may be by a factor of 10, and between the second and third amounts may be a factor of 5. In another example, the reduction in between first and second amounts may be by a factor of 2 and between second and third amounts may be by a factor of 5.

The method of screening a phage display library may (where the phage display library contains antibody sequences) be followed by the further step of producing a complete antibody. Producing a complete antibody may comprise insertion of the antibody sequences isolated from the phage display library into a suitable vector (such as pLNOH2 and pLNOK, as described herein) and expressing the antibody from that vector in an appropriate cell type.

Antibodies produced or producible by such a method represent a further aspect of the invention.

Uses of antibodies

The antibodies disclosed herein can be used for treatment or for prevention of a malarial disease.

The term "malarial disease" includes clinical, e.g. clinical symptomatic infection, clinical asymptomatic infection and cerebral malaria.

Accordingly, in a further aspect the present invention provides recombinant human antibodies disclosed herein for use in the treatment or prophylaxis of a malarial disease.

The invention further encompasses the use of recombinant human antibodies disclosed herein in the manufacture of a medicament (e.g. a vaccine) for the treatment or prophylaxis of malaria. The medicament may further comprise a suitable excipient or carrier.

Suitable excipient, carriers, buffers, stabilises are well known to those skilled in the art. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, or Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Methods of treatment or prophylaxis of malaria form further aspects of the invention, such methods may comprise administering a recombinant human antibody as described herein to an individual.

In the above methods and uses, the recombinant antibodies may be administered to the individual in any of the following ways:

intravenous injection; intramuscular injection; sub-dermal
injection; oral administration e.g. using capsules to protect
the antibody during passage to the stomach; administration in
toothpaste (whereby the antibody is transferred to the

5   circulation by the abrasive affects in the oral mucosa);
administration by the use of occlusive plasters or skin ointment
for penetration through the skin; administration through the
nasal mucous by delivery with a spray; and rectal
administration.

10

Suitable doses for treatment may be 1 mg to 10 mg of antibody
per kg body weight or less depending on the affinity of the
antibody, preferably 1.5 mg to 3.5 mg, most preferably 2 mg
antibody per kg body weight.

15

Suitable doses for prevention may be smaller due to the lower
load of parasites to be eliminated, such as 0.1 μg to 3 mg,
preferably 0.1 mg to 2 mg, more preferably 0.5 mg to 1.5 mg,
more preferably 0.75 mg to 1 mg most preferably 1mg antibody per

20   kg body weight.

Suitable administration schemes may be based upon an *in vivo*
half-life for the antibodies of the IgG1 class of around to 23
days and an *in vivo* half-life for the IgG3 class of around 6

25   days.

Alternatively, or additionally, the nucleic acid molecule
encoding the antibody may be used in the treatment or prevention
of malarial disease. In this way the DNA is injected into the

30   individual and the antibody is produced endogenously in that
individual.

Accordingly, further aspects of the invention relate to: the
nucleic acid molecules described herein for use in the treatment

35   or prophylaxis of a malarial disease; the use of nucleic acid
molecules described herein in the manufacture of a medicament
(e.g. a vaccine) for the treatment or prophylaxis of malaria;
and methods of treatment or prophylaxis of malaria comprising

administering a nucleic acid molecule as described herein to an individual.

Suitable vectors for the administration of nucleic acid molecules to an individual include adenovirus and adeno-associated virus, and retrovirus vectors, in addition to other particles that introduce DNA into cells, such as liposomes.

A further aspect of the present invention provides a method which comprises causing or allowing binding of an antibody as provided herein to a target antigen.

Such binding may take place *in vivo*, e.g. following administration of an antibody, or nucleic acid encoding a antibody, or it may take place *in vitro*, for example in ELISA, Western blotting, immunocytochemistry, immuno-precipitation or affinity chromatography.

In vivo, this may be useful for vaccine where the antibodies disclosed herein may serve as an adjuvant to enhance the endogenous immune response. Alternatively, the antibodies disclosed herein may serve as transport molecule facilitating the uptake of the MSP-3 antigen, e.g. from the intestinal tract (for this latter function IgA is preferred).

The amount or extent of binding of specific binding member to a target antigen may be determined. Quantitation may be related to the amount of the antigen in a test sample, which may be of diagnostic interest.

The reactivities of specific binding members on a sample may be determined by any appropriate means. Radioimmunoassay (RIA) is one possibility. Radioactive labelled antigen is mixed with unlabelled antigen (the test sample) and allowed to bind to the specific binding member. Bound antigen is physically separated from unbound antigen and the amount of radioactive antigen bound to the specific binding member determined. The more antigen there is in the test sample the less radioactive antigen will bind to the specific binding member. A binding assay may also

be used with non-radioactive antigen, using antigen or an analogue linked to a reporter molecule. The reporter molecule may be a fluorochrome, phosphor or laser dye with spectrally isolated absorption or emission characteristics. Suitable fluorochromes include fluorescein, rhodamine, phycoerythrin and Texas Red. Suitable chromogenic dyes include diaminobenzidine.

The present invention also provides the use of a specific binding member as above for measuring antigen levels in a competition assay, that is to say a method of measuring the level of antigen in a sample by employing a specific binding member as provided by the present invention in a competition assay. This may be where the physical separation of bound from unbound antigen is not required. Linking a reporter molecule to the specific binding member so that a physical or optical change occurs on binding is one possibility. The reporter molecule may directly or indirectly generate detectable, and preferably measurable, signals. The linkage of reporter molecules may be directly or indirectly, covalently, e.g. via a peptide bond or non-covalently. Linkage via a peptide bond may be as a result of recombinant expression of a gene fusion encoding antibody and reporter molecule.

Thus a further aspect of the invention is a method of diagnosis of malaria. Such a method may comprise taking a sample of bodily fluid from an individual, contacting the sample with an antibody as described herein and determining the binding of that antibody to the sample, thereby determining the presence or absence of a target antigen in the sample. Thus the binding of an anti-MSP-3 antibody described herein would show the presence of the MSP-3 antigen in the sample.

In addition, antibodies according to the invention may be used in methods to assist in the identification or production of molecules with anti-parasitic, e.g. anti *Plasmodium falciparum* activity. In this way, antibodies may be used to guide the selection of novel antibodies against the MSP-3 antigen or cross reactive antigen (Ohlin et al, 1996; Jirholt et al, 1998), or the antibodies may be used to identify the single components in

the protective mechanisms responsible for the anti-parasitic effects and to identify other substances enhancing or interfering with these effects.

5   The invention will now be described in detail with reference to the following figures and examples, which are non-limiting.

BRIEF DESCRIPTION OF THE DRAWINGS AND TABLE

10   Figure 1 shows ELISA reactivity with various truncated versions of MSP-3. Fab-ΔpIII fragments produced from the three distinct clones were analysed in ELISA. Truncated recombinantly produced, MSP-3_{22-257} (black bars), MSP-$3_{194-257}$ (grey bars) , were used as coating antigens in separate analyses. An ELISA for
15   detection of Fab fragments (Anti-Fab) was included to ensure use of comparable amounts of the various Fab fragments (white bars). Background was measured as reactivity with the control Fab against HibCP and as reactivity with buffer. Reactivities are indicated as $OD_{405}-OD_{490}$. Bars indicated median values and error-
20   bars indicated 2 times standard deviation of triplicates.

Figure 2 shows results of the antigen competition experiments. Fab-ΔpIII from the three distinct clones were competed with soluble panning antigen in ELISA. All three clones were
25   susceptible to competition with soluble MSP-$3_{194-257}$. A constant amount of Fab-ΔpIII produced from each of the three distinct clones was mixed with varying amounts of competition antigen, MSP-$3_{194-257}$, and applied to ELISA wells coated with the same antigen. Percent reactivity in the ELISA was calculated taking
30   the reactivity with no antigen added as 100%.

Figure 3 shows a schematic representation of the entire MSP-3 antigen (top, MSP-3_{1-380}) and the relationship of the truncated antigens (MSP-3_{22-257}, MSP-$3_{194-257}$, MSP-$3_{190-217}$ and MSP-$3_{211-237}$ used).
35   The numbering above has been assigned according to the P. falciparum clone D10, sequenced by McColl et al. 1994 (Genbank accession number L07944). As this numbering may be different when aligned with other clones, the MSP-$3_{194-257}$ (Oeuvray et al.

1994, accession number AF024624) amino acid sequence has been
written in the figure.

The intact antigen comprises 12 heptad repeats, equally
distributed into the three heptad repeat regions (HELIX 1, HELIX

5   2, HELIX 3). Analyses of the sequence of the regions suggest
that they have an amphipatic α-helical secondary structure and
in the presence of all three regions can obtain the structure of
a coiled-coil three-stranded helical bundle (McColl et al. 1994;
Mulhern et al. 1995). The helix regions and signal peptide

10   (SIGNAL) are indicated as boxes at the top of the drawing. The
dimorphic areas described by Huber et al. (1997) are indicated
with gray shading. The $MSP-3_{190-217}$ peptide represents the Helix
3 sequence of the K1 clone (McColl et al. 1997, accession number
U08851).

15   The motif represented by amino acid residues 220-230,
ILGWEFGGGVP, is indicated by oval figures. This motif is
present in MSP-3 as well as in the other *Plasmodium falciparum*
antigen MSP-6 (Trucco et al. 2001).

20   Figure 4 shows the results of the flow cytometry analyses of the
reactivity of recombinant antibodies with schizonts. Panel A1
illustrates forward scatter and side scatter for ethanol-fixed
and – permeabilized infected red blood cells, and panel A2
illustrates the same parameters for similarly treated non-

25   infected red blood cells. The gating was placed to include the
majority of the infected cells. The gating was used for the
histograms described below.

Panel B1 to 4 illustrates histograms of various recombinant anti-
malaria antibodies compared with the control antibody directed

30   against HibCP antigen (grey shaded curve). The control histogram
has 2% of the events under the bar designated M1 in panel B1.

B1 shows RAM1 in the Fab–ΔpIII format. The bar covers 76% of the
events registered.

B2 shows RAM2 in the Fab–ΔpIII format. The bar covers 28% of the

35   events registered.

B3 shows RAM3 in the Fab–ΔpIII format. The bar covers 4% of the
events registered.

B4 shows RAM1 in the IgG1 format. The bar covers 47% of the
events registered. The control in the IgG1 format has 4 % of the
events under the bar.

Schizonts were purified from asynchronous *P. falciparum* culture.
Fixation and permeabilization was carried out with ethanol.
Primary reaction with recombinant antibody was carried out over
night and the FITC-labeled secondary antibody was incubated with
the cells for 30 minutes.

Figure 5 shows a schematic representation of the panning
procedure. Three parallel series (A to C) of panning were
carried out for 4 rounds designated 1 to 4. The number of
antigen-coated beads used in each round was reduced by a
constant reduction factor through the study. The number of
beads used for each stage is indicated in the figure.

Figure 6 shows the deduced amino acid sequences of the three
clones. The entire VH and VK sequences of each clone, RAM1,
RAM2 and RAM3 are shown and boxed. Amino acid residues in the
CDR regions are underlined. We have chosen to show the Kabat
CDR definitions (Kabat et al. 1991).

Figure 7 shows the immunoblotting of *P. falciparum* clone 3D7
schizont and merozoite proteins reacted with RAM1 and RAM2
produced as IgG1. Both lanes show a blotting of proteins from
purified schizonts separated under reducing conditions on a 4-12%
gradient gel. RAM1 IgG1 was reacted with the blotting in lane 1
and RAM2 IgG1 was reacted with the blotting in lane 2.

RAM1 recognizes two proteins of approximate relative molecular
weights 51 and 53 kD, respectively.

RAM2 reacts with two proteins of approximate relative molecular
weights of 64 kD and 14 kD, respectively.

Figure 8 shows Immunofluorescence microscopy. Panel *a*
represents immunofluorescence microscopy of fixed culture of
P.falciparum clone 3D7 incubated with RAM1, Fab-ΔpIII followed
by a FITC conjugated secondary anti-human Fab. Dots in panel *a*
indicate red cells carrying antigen reactive with RAM1. Panel *b*
represents the slide stained for DNA with propidium iodide.

Large dots in panel *b* indicate red cells infected with late
stage schizonts. By comparing the location of the dots in the
two panels it is observed that the larger dots in panel *b* is
duplicated in panel *a*. Small dots in panel *b* is not duplicated
5   in panel *a*. This demonstrates that late-stage schizonts
harbouring merozoites do display the antigen reactive with RAM1
whereas early stages do not. Asynchronous in vitro *P.
falciparum* culture was air-dried on a slide, fixed with acetone
and reacted with RAM1 followed by FITC-conjugated anti-human
10   Fab. The other two clones, RAM2 and RAM3, both gave an
identical pattern of reactivity.

Figure 9 shows the ELISA reactivity of RAM1 IgG1 and RAM2 IgG1
with the synthetic peptides MSP-$3_{190-217}$ and MSP-$3_{211-237}$.
15   Also the larger recombinant MSP-$3_{194-257}$ has been included.

Figure 10 shows the results from the PEPSCAN screening.
The entire sequence MSP-$3_{194-257}$ was covered by synthesis of 34
sets of 14-mer and 15-mer peptides comprising contiguous amino
20   acid sequences. The two peptides were linked by a proprietary
linker substituting two amino acid residues on the location of
the linker (PEPSCAN). Thus one single set of peptides covers a
stretch of 31 amino acid residues with two amino acid residues
replaced in positions 15 and 16 by the proprietary linker
25   residues. The linked peptides were furthermore chemically
coupled to the matrix to allow washing and repeated use. The
overlap between to adjacent sets of peptides was two amino acid
residues. This collection of peptides was examined for
reactivity with RAM1 and RAM2 produced as IgG1. RAM2 reacts
30   with the amino-terminal, the middle and the carboxy-terminal
part of the antigen but yields the highest reactivity with the
middle part. RAM1 reacts only weakly with the peptides produced
by this method. RAM3 was tested as Fab-ΔpIII in PEPSCAN without
response.

35

Figure 11 shows the inhibitory effect of immune serum on the
binding of RAM1 IgG1 to MSP-$3_{194-257}$. The FITC-conjugated RAM1
IgG1 antibody was applied to ELISA wells coated with MSP-$3_{194-257}$
were pre-incubated for two hours with dilutions of immune or

negative control serum and then a constant amount of FITC-conjugated RAM1 IgG1 antibody was applied to each well. Immune serum blocked the binding of RAM1.

5　Figure 12 Construction of expression vectors. The V-regions of the plasmids can be exchanged by cutting with *BsmI* in the 5' end and by cutting with *HpaI* or *BsiWI* or *HindIII* in the 3' end. Before ligation of vector and V region, the V regions of RAM1, RAM2 or RAM3 need to be cut with the same restriction enzymes as

10　the vectors. VH regions are then ligated to pLNOH2 and VK regions are ligated to pLNOK. The constant region of pLNOH2 can be exchanged by cutting with *BamHI* and *HindIII*, isolating the vector part and introducing (by ligation) another constant region cut with the same enzymes.

15　In order to produce a complete antibody the plasmids (both pLNOH2 and pLNOK) are co-transfected into CHO cells. The V and C-regions are assembled by mRNA splicing. Alternatively the genes can be assembled into one vector thus harbouring the VH plus CH and VL plus CL. This insures that the two genes are

20　present in equimolar numbers.

Abbreviations: CMV = CMV promoter, L = leader, VH = variable heavy, VK = variable kappa, CH = constant heavy chain gene, CK = constant kappa chain gene, BGHpA = Bovine Growth Hormone polyadenylation site, f1 = f1 origin, SV40ori = SV40

25　origin/promoter Neo = gene for neomycin resistance, Amp = gene for ampicillin resistance

Figure 13 Antibody competition. Fab fragments from clone RAM3 were produced in two versions. One version was fusion protein

30　tagged with a truncated phage protein, ΔpIII, designated Fab-ΔpIII, and another version was normal Fab fragments without the ΔpIII tag. The ΔpIII-tag is readily detected using an antibody directed to the phage pIII protein. Thus Fab without ΔpIII can be present as competitor but only Fab-ΔpIII will be detected.

35　Amounts of RAM1, RAM2 and RAM3 Fab-ΔpIII yielding OD_{405} of approximately 1 in ELISA on a coating of $MSP-3_{194-257}$ were used. The binding of the Fab-ΔpIII was then competed by the addition of increasing amounts of competitor, RAM3 without ΔpIII. This

figure shows that Fab RAM3 is able to compete Fab-ΔpIII RAM1 to the same extent as it competes Fab-ΔpIII RAM2. This demonstrates that binding of the clones RAM1 and RAM2 is dependent on the epitope used by clone RAM3. The lower

5   competitive effect of RAM3 on the binding of Fab-ΔpIII RAM3 could be explained by a higher affinity of RAM3 as compared to RAM1 and RAM2. The effect of this would be that the competitive effect of RAM3 towards RAM1 and RAM2 would be larger than towards RAM3 itself.

10

Figure 14 shows the organization of heavy and light chain genes in pLNOH2 and pLNOK, respectively, and the resulting antibody structure. The part of an antibody responsible for binding to antigen is the variable region, the V-region. This region

15   consists of two separate polypeptides. One polypeptide comes from the light chain and the other from the heavy chain. In the intact antibody the variable region polypeptide from the light chain extends into the constant domain of the light chain, thus giving rise to an approximately 200 amino acid residues long

20   polypeptide with a total of two domains. In the intact antibody the variable region from the heavy chain extends into the constant domains 1 to 3 giving rise to a 400 amino acid residues long polypeptide with a total of four domains. In the intact antibody two heavy chains combine with two light chains. Thus

25   an intact antibody contains two V-regions and is able to bind to two antigens at the same time. The constant regions of the heavy chain interacts with the effector functions of the immune system.

30   Table I shows the results of screening of single clones from the three series after the fourth panning.

DETAILED DESCRIPTION OF THE INVENTION

35   Example 1   Library construction

Sampling of peripheral blood lymphocytes

A volume of 100 ml peripheral blood was obtained after informed consent from each of 13 adults living in a malaria endemic area of Senegal, West Africa. The blood was collected in the anticoagulant ACD (adenine-citrate-dextrose) that conserves RNA
5 better than heparin. The samples were transported to the laboratory where a brief centrifugation was carried out, the buffy coat aspirated and cells suspended into an RNAse protecting buffer containing guanidinium HCl, beta-mercaptoethanol and sarcosine. The material was then frozen in liquid nitrogen and
10 shipped for further processing.

RNA purification

RNA was isolated from the samples by acid phenol extraction
15 according to the procedure of Chirgwin et al. (Chirgwin et al. 1979). Messenger RNA was converted to cDNA as described by Ørum et al. (1993).

PCR of antibody coding genes

20

Amplification of antibody genes was carried out as described previously (Dziegiel et al. 1995). Briefly, separate reactions were performed for the V_H genes, lambda light chain genes, and for the kappa light chain genes. A pool of 12 individual primers was
25 used for priming in the 5' region of the V_H genes (backward primers) and a pool of 3 primers was used for priming in the J_H region (forward primers). The details of the primers are given in Dziegiel (1995).

30 The amplification product of approximately 350 bp was purified from a low-melting point agarose by digestion of the agarose with agarase (Boehringer-Mannheim, Germany). This fragment was used as a template for a secondary extension PCR with primers complementary to the ends of the primary product and additionally
35 containing sequences corresponding to the restriction enzymes *Nhe*I (5') and *Apa*I (3'). Extended V_H fragments thus have a 5' *Nhe*I site and a 3' *Apa*I site. A pool of 8 primers was used for priming in the 5' region of kappa light chain genes and one primer for priming in the 3' region. For amplification of lambda

light chain genes a pool of 12 forward primers and one backward primer were used. The resulting fragment of approximately 700 bp was purified as above and used as template for a secondary extension PCR introducing the restriction enzyme sites *Sfi*I (5')

5 and *Asc*I (3'). Extended kappa chain genes thus have a 5' *Sfi*I site and a 3' *Asc*I site.

Polymerase chain reaction

10 Antibody genes were amplified from cDNA by PCR with separate reactions for V_H region genes, λ chain genes, and κ chain genes, respectively. PCR was performed in 100 µl volumes containing 0.2 mM dNTP's and reaction buffer supplied by the manufacturer (HT Biotechnology, UK), an equimolar mixture of primers totalling 20

15 µM (i.e. 0.65 µM of each V_H primer, 0.74 µM of each Vκ primer, 0.83 µM of each V_λ primer, 20 µM of the κ chain constant domain primer, 5 µM of each of the λ chain constant domain primer, and 5 µM of each of the J-region primers), cDNA, and enzyme. An initial one min period at 94°C was followed by addition of

20 enzyme, and the cycle (94°C for one min, 55°C for one min, 72°C for one min) was then repeated 30 times. Reactions were performed as described (Ørum et al., 1993), except that a HYBAID Omnigene thermocycler was used.

Plasmid pFAB73HHui

The pFAB73HHui vector is developed from pFAB73H (Dziegiel et al.,
1995; Engberg et al., 1996). The vector harbors an intact *lacI*^{q}
5   gene encoding a highly efficient mutant of the *lacI* repressor.
This ensures a high level of gene repression independent of the
bacterial background. The sequences between the *NheI - ApaI*
sites, and between the *SfiI - AscI* sites have been exchanged with
sequences originating from a murine antibody against human
10   insulin. This improves the efficiency of the second digestion
and facilitates isolation of double digested plasmid during the
subsequent low-melting point agarose electrophoresis. Other
features of the plasmid are identical to the pFAB4H. Briefly,
pFAB73HHUI contains the DNA fragment encoding amino acid residues
15   118 to 230 of the human γ1 H chain corresponding to constant
domain 1 (C_H1). The C_H1 gene extends in frame into a truncated
version of *gene III* (ΔgIII) via a DNA fragment encoding a spacer
and a trypsin cleavage site. ΔgIII encodes a truncated version of
surface protein pIII (ΔpIII) of the filamentous phage, f1 (Bass
20   et al., 1990; Ørum et al., 1993). The lacZ promoter and the 5'
part of the PelB leader gene were positioned in front of cloning
sites for L chain genes. A DNA fragment containing a ribosome
binding site (RBS) and the 5' part of the gene encoding the PelB
leader was positioned 5' to cloning sites for H chain variable
25   region (V_H) genes.

pFAB73HHUI permits insertion of V_H genes and L chain genes by a
two-step cloning procedure, first inserting V_H genes into
restriction enzyme sites NheI and ApaI, then inserting L chain
30   genes into the sites SfiI and AscI. All necessary genetic
elements and the C_H1 are present in the vector, thus minimizing
the total number of PCR cycles.

Cloning of antibody genes

35

Amplification products from the secondary extension PCRs were
separated on low-melting point agarose followed by digestion with
agarase. The extended V_H fragments were digested with restriction
enzymes *NheI* and *ApaI* in two sequential reactions. The *NheI*-

*Apa*I-digested V_H gene fragment was purified on a low-melting point agarose gel followed by agarase digestion. The cloning vector, pFAB73HHUI, was digested with restriction enzymes *Nhe*I and *Apa*I in two sequential reactions. The *Nhe*I-*Apa*I-digested 5.4 kb

5   plasmid was purified on a low-melting point agarose gel followed by agarase digestion. The V_H gene was ligated into the plasmid, the ligation mixture was extracted with Phenol and Chloroform, ethanol precipitated and electroporated into *E. coli* Top10Tet using an E. coli pulser set (BioRad). Bacteria were resuspended

10   in SOC medium (Sambrook et al. 1989), incubated at 37°C for 1 h with shaking and then plated on LB agar (Sambrook et al. 1989) containing 50 mg/l carbenicillin, 12.5 mg/l tetracycline and 2% glucose. Plates were incubated overnight at 37°C. Colonies were washed off the plates with LB medium with carbenicillin,

15   tetracycline and glucose and used to start a liquid culture, which at $OD_{600} = 1$ was used to prepare the recombinant vector using Qiagen columns (Qiagen GmbH, Hilden, Germany) according to the procedures recommended by the manufacturer. The purified vector containing H chain genes was used as cloning vector for

20   the cloning of kappa and lambda light chain genes.

The extended kappa chain products were digested with restriction enzymes *Asc*I and *Sfi*I in two separate reactions. The kappa chain genes were subsequently cloned into SfiI- and AscI-digested

25   pFAB73HHUI harbouring V_H region genes.

Library

The resultant library contained 5×10^7 different heavy chains,

30   1×10^8 kappa light chains and 1×10^8 lambda light chains. Investigation of V_H genes from 66 clones by PCR and *Bst*NI digestion demonstrated lack of identical digestion patterns thus suggesting acceptable diversity.

35   In this system, the heavy (H) chain fragment, Fd, is produced as a fusion protein with a truncated version of the filamentous f1 phage protein III (ΔpIII) (Bass ET al. 1990). H chain fusion proteins associate with intact light (L) chains in the

periplasmic space to form Fab heterodimers, which are fused to the ΔpIII protein. Upon infection with helper phage, the fusion proteins are positioned on one end of the phage together with a few copies of helper phage-derived wild-type pIII (Clackson et al. 1991; Hoogenboom et al. 1991). Thus, the Fab is able to bind to its specific antigen, and at the same time remains physically associated with the phagemid DNA which harbors the genes encoding that Fab, i.e. a physical linkage has been established between recognition and replication. This allows selection of a given antibody specificity and its corresponding gene from a large number of different Fab-phage (Marks et al. 1991).

Example 2- Selection

Production of primary phage stock

A 50 ml culture of the library in LB medium with 50 mg/l carbenicillin, 12,5 mg/l tetracyclin and 2% glucose was superinfected with VCSM13 helper phage (Stratagene) at an OD_{600} = 0.8. A multiplicity of infection of 100 was used and the mixture was incubated at 37°C with gentle shaking (50 rpm) for 1 h. Then the culture was diluted into 950 ml medium as above without glucose and incubated at 30°C overnight. After a 15-min spin at 10,000 xg the supernatant containing phage was precipitated with PEG_{6000} and sodium chloride at final concentrations of 4% and 0.5 M respectively. The supernatant was incubated for 1 h on ice and centrifuged for 30 min at 12,000 x g. Precipitated phage was resuspended in phosphate buffered saline (PBS) with 0.1% bovine serum albumin (BSA) and used immediately. The total number of colony forming units was determined as 1.3×10^{13} (Sambrook et al. 1989).

Selection of specific binders in the first panning

Biotinylated MSP-$3_{194-257}$ was coupled to Dynabeads M-280
Streptavidin (cat no. 112.05) as described by the manufacturer.
5   After incubating the antigen-coated beads with phage in an end-
over-end mixer for 1 h, beads were captured with a magnetic
particle concentrator (MPC-6, Dynal Cat no. 120.02) for 5 min.
and then washed 6 times in 10 ml PBS with 0.05% Tween20 for 2
min.   After the last wash beads were resuspended in 1 ml PBS
10   with 1 mg trypsin (Worthington, USA) and phage was eluted during
a one-hour incubation at 37°C.   Then a volume of 3 ml of
exponentially growing Top10 with OD_{600} = 1 was added and the
mixture was incubated at 37°C for 30 minutes to allow eluted
phage to attach to E. coli.   The bacteria were finally plated on
15   LB agar (Sambrook et al. 1989) containing 50 mg/l carbenicillin,
12.5 mg/l tetracyclin and 2% glucose and incubated overnight at
37°C.   Colonies were washed off the plates with LB medium and
stored as a glycerol stock at -80°C or grown for production of
phage and DNA.   DNA was produced as described.

20

Production of phage for subsequent pannings

Phage was produced by super-infecting a 15 ml culture of the
glycerol stock at OD_{600} = 0,6 (after approx. 3 hours) by addition
25   of helper phage VCSM13 (Stratagene, USA).   Multiplicity of
infection was approx. 100.   After 1 hour of gentle shaking the
culture was diluted into 200 ml of LB-medium and supplemented
with isopropyl-β-D-thiogalactopyranoside (IPTG) to give a final
concentration of 50 μM, 70 mg/l kanamycin, 50 mg/l Carbenicilin
30   and 12,5 mg/l tetracyclin.   The culture was grown over night.
Phage was isolated as above except that it was cleared before
panning by two additional spins of each 20 minutes at 17,320xg.

Selection of specific binders in subsequent pannings

35

Subsequent to the first panning, three series of panning were
carried out in parallel, to a total of 4 rounds.   In the first
series, the number of beads was reduced by a factor of 5 for each

subsequent panning. In the second and the third series the number of beads was reduced by a factor of 10 and 20, respectively, from one panning to the next. After the fourth panning single colonies from all three series were grown in sterile microtiter plates for production of Fab-ΔpIII.

Testing of final eluate

The eluate from the fourth panning was screened for the presence of Fab-ΔpIII producers and antigen binders, respectively. Fab-ΔpIII was produced by growing individual colonies in microtiter wells. A total of 376 clones were tested. Supernatant from 23 clones contained free Fab-ΔpIII and 17 of these were able to bind to MSP-$3_{194-257}$ (see table I). The antibody genes of the 17 clones were sequenced using Big Dye according to the procedures of the manufacturer (Applied Biosystems, USA).

The selection procedure resulted in isolation of three distinct clones designated RAM1, RAM2 and RAM3.

Example 3 – Determination of Specificity

The reactivity of the clones with native malaria antigen was demonstrated.

Direct ELISA

ELISA plates (Maxisorb, NUNC 4-39454, Denmark) were coated with 10 to 650 ng per well of purified recombinant MSP-3_{22-257} or MSP-$3_{194-257}$ $_{257}$ or of the peptides MSP-$3_{190-217}$ or MSP-$3_{211-237}$ in PBS and used for standard ELISA with undiluted supernatant or supernatant diluted in PBS-BSA as previously described (Dziegiel et al.1991; Dziegiel et al. 1995). After wash, goat anti-human Fab (Sigma A8542) or anti-human IgG Fc (Sigma A9544) antibodies conjugated to alkaline phosphatase was applied as detection antibody. Finally, p-nitrophenyl phosphate (Sigma phosphatase substrate tablets, 104-105) was used as substrate. Color development was measured as OD$_{405}$ – OD$_{490}$, see Figure 1. RAM1 and RAM2 both bind

to the short MSP-$3_{194-257}$ and the long MSP-3_{22-257} recombinant versions of the MSP-3 antigen. In contrast, RAM3 binds only the short MSP-$3_{194-257}$ version. The controls (anti-Hib CP and PBS 1% BSA) do not react with the antigen.

Antigen competition

To wells containing 50 μl of a fixed dilution of Fab-ΔpIII were added 50 μl of various dilutions of competition antigen, MSP-$3_{193-256}$. Coating of wells with MSP-$3_{194-257}$ and detection of bound antibody were carried out as above. The concentration of competition antigen ranged from 4 nM to 1250 nM. Each dilution of competition antigen was tested in duplicate, see Figure 2. All three clones are competed by soluble recombinant MSP-$3_{194-257}$ antigen. This demonstrates that the antibodies are directed to the conformation of the antigen in solution and not to some denaturation-dependent conformation e.g. a plastic-binding dependent epitope.

Immunofluorescence microscopy

Parasitized erythrocytes from in vitro culture of *P. falciparum* clone 3D7 were attached to a slide, air dried and then fixed with acetone for 5 minutes at room temperature as described (Druilhe et al. 1987). Fab-ΔpIII or intact IgG1 was added and after 30 minutes slides were washed in PBS. Bound Fab-ΔpIII was detected with FITC-conjugated goat anti-human Fab (Sigma F5512) diluted 1:25. Hib-CP specific antibody was used as control in corresponding concentrations. To stain the DNA-containing infected erythrocytes propidium iodide was added to the secondary antibody solution. The late schizont stage was intensively stained with propidium iodide due to its high content of merozoite DNA. The RAM1 Fab stained exactly this stage. In contrast, red cells containing small amounts of parasite DNA did not bind to the RAM1 Fab. Red cells with small amounts of DNA represents early stages, i.e. ring stage and trophoblasts, see Figure 8.

Immunoblotting

Purified parasites were solubilized in 2% Triton X-100 by
treatment with ultrasound 4 x 15 seconds on ice and incubation
for 2 hours on ice before being mixed with sample buffer, heated
to 100°C for 10 minutes and subjected to SDS-PAGE in a MOPS-
buffered 4-12% gradient gel (NOVEX). Proteins were transferred
electrophoretically to a PVDF membrane (Immobilon, Millipore) by
wet blotting, blocked by drying the membrane and by incubation
in 0,1 % Tween20 and 0,2 % I-Block (Tropix Cat. No. AI300)
immediately before use. The blot was incubated for 3 hours with
anti-malaria antibody RAM1 or RAM2 produced as IgG1, washed 3x5
minutes with PBS supplemented with 0.1% Tween20 and 0,2 % I-
Block, and then incubated for 1 hour with alkaline phosphatase
conjugated protein G (Pierce Cat no.32391). The membrane was
washed 3 times as above. Finally, CSPD chemiluminescent
substrate (Tropix Cat. no.CD100R) was added to the blot and
light emission was detected with the Alpha Innotech FluorChem
8000 system. Hib-CP specific antibody was used as negative
control in corresponding concentrations, see Figure 7. RAM1
reacted with parasite proteins of relative molecular weights of
approximately 51 and 53 kD, respectively. RAM2 reacted with
parasite proteins of relative molecular weights of approximately
14 and 64 kD, respectively. Molecular weights in the same range
have been observed with polyclonal antibodies from man and
rabbit (Oeuvray et al, 1994; McColl et al, 1994). The
background for the variation in molecular weights of the bands
recognised by these two antibodies could be post-translational
modification, proteolytic processing or the presence of
subpopulations of molecules with distant conformations. The
latter possibility is tempting due to the alleged propensity of
MSP-3 to obtain intra- or intermolecular coiled-coil
conformations (McColl et al, 1994) later extended by Trucco et
al (2001) to pertain to other merozoite specific proteins like
MSP-1 and MSP-6. Such tertiary conformation will influence the
electrophoretic mobility and could thus be responsible for the
observed range of molecular weights. The band of 14 kD is
believed to be due to proteolytic degradation. There have been
no other reports of bands in this range.

Flow Cytometry

Purified schizonts were permeabilized and fixed by incubation in
32% ethanol for 30 minutes on ice. Parasites were then washed in
PBS with 1% BSA, incubated over night with antibody (Fab-ΔpIII or
IgG1), washed twice with PBS supplemented with 1% BSA and
incubated for 30 minutes with FITC-conjugated goat anti-human Fab
(Sigma F5512) diluted 1:25. Cells were analyzed in a Coulter
EPICS-2 flow cytometer. Hib-CP specific antibody was used as
control in corresponding concentrations. Fixed infected and
fixed non-infected red cells were stained with propidium iodide
and compared to enable gating on infected red cells, see Figure
4.

Epitope mapping

Our mapping of the epitopes relates to a previously used
arbitrary division of MSP-3$_{194-257}$ into three peptides (Oeuvray et
al. 1994) termed MSP-3a, MSP-3b and MSP-3c (Oeuvray et al.
1994). The biological relevance of the dissection of MSP-3$_{194-257}$
is that polyclonal antibodies directed to MSP-3b, corresponding
to MSP-3$_{211-237}$, show a clear anti-parasitic effect in ADCI and in
the mouse model (Badell et al. 2000).

Two peptides, MSP-3$_{190-217}$ and MSP-3$_{211-237}$, were synthesized and
used in ELISA. RAM1 reacts predominantly with MSP-3$_{211-237}$. RAM2
reacts to the same extent with MSP-3$_{190-217}$ and MSP-3$_{211-237}$, see
Figure 9.

Additionally, the entire sequence MSP-3$_{194-257}$ was covered by
synthesis of 34 sets of two peptides comprising contiguous amino
acid sequences. The two peptides were linked by a proprietary
linker substituting two amino acid residues on the location of
the linker (PEPSCAN). Thus one single set of peptides covers a
stretch of 31 amino acid residues with two amino acid residues
replaced in positions 15 and 16 by the proprietary linker
residues. The linked peptides were furthermore chemically
coupled to the matrix to allow washing and repeated use. The

overlap between two adjacent sets of peptides was two amino acid residues. This collection of peptides was examined for reactivity with RAM1 and RAM2 produced as IgG1. The result is presented in Figure 10. RAM2 reacts with the amino-terminal,

5   the middle and the carboxy-terminal part of the antigen but yields the highest reactivity with the middle part. RAM1 reacts only weakly with the peptides produced by this method. RAM3 was tested as Fab-ΔpIII in PEPSCAN without response.

10 The interpretation of the results is that RAM1 as well as RAM2 react with conformational, non-linear epitopes comprising amino acids residues being non-contiguously positioned in the antigen.

The epitope for RAM1 certainly comprises residues in the middle
15 part. The contribution of residues in the amino-terminal and the carboxy-terminal part of the antigen is uncertain. The amino-terminal part of the antigen represented by the peptide MSP-$3_{190-217}$ comprises only heptad repeat sequence. The first residues of MSP-$3_{211-237}$ are part of a heptad repeat making it
20 likely that the epitope for RAM1 is the c-terminal part of MSP-3_{211}-237. The region represented by residues 220-230, ILGWEFGGGVP, is outstanding by being present in MSP-3 as well as the other *Plasmodium falciparum* antigen MSP-6 (Trucco et al. 2001).

25

Based on the PEPSCAN results we conclude that RAM2 reacts with residues from all parts of the antigen but the major contribution is from the middle part.

30 *Antibody Competition*
The studies of binding to truncated antigens were supplemented with an antibody competition experiment. Fab fragments from clone RAM3 were produced in two versions. One version was fusion protein tagged with a truncated phage protein, ΔpIII, designated
35 Fab-ΔpIII, and another version was normal Fab fragments without the ΔpIII tag. The ΔpIII-tag is readily detected using an antibody directed to the phage pIII protein. Thus Fab without

ΔpIII can be present as competitor but only Fab-ΔpIII will be detected.

Amounts of RAM1, RAM2 and RAM3 Fab-ΔpIII yielding OD_{405} of approximately 1 in ELISA on a coating of MSP-$3_{194-257}$ were used. The binding of the Fab-ΔpIII was then competed by the addition of increasing amounts of competitor, RAM3 without ΔpIII. Figure 13 shows that Fab RAM3 is able to compete Fab-ΔpIII RAM1 to the same extent as it competes Fab-ΔpIII RAM2. This demonstrates that binding of the clones RAM1 and RAM2 is dependent on the epitope used by clone RAM3. The lower competitive effect of RAM3 on the binding of Fab-ΔpIII RAM3 could be explained by a higher affinity of RAM3 as compared to RAM1 and RAM2. The effect of this would be that the competitive effect of RAM3 towards RAM1 and RAM2 would be larger than towards RAM3 itself, see Figure 13.

RAM3 was only reactive with the intact panning antigen and did not react with truncated versions. Furthermore RAM3 did not react with parasites in flow cytometry. However, the antibody competition experiment above shows that the epitopes for RAM1, RAM2 and RAM3 are closely dependent be it by direct steric hindrance due to overlap of epitopes or an effect of binding of RAM3 on the correct folding of the antigen.

We then tested if a malaria immune serum obtained from an individual living in a malaria-endemic area contains antibodies that will interfere with the binding of clone RAM1 to MSP-$3_{194-257}$. Clone RAM1 was produced as IgG1 and conjugated to FITC. The FITC-IgG1 was used in an assay identical to ELISA except for the detection of binding that was carried out by measurement of fluorescence in a microtiter plate based fluorometer. The amount of FITC-IgG1 was adjusted to give maximum effect of competition. Increasing amounts of competitor immune serum was added to individual well of the microtiter plate. A serum from a non-immune European donor was used as negative control. The malaria immune serum contained antibodies that significantly

inhibited the binding of RAM1 IgG1. For technical details see below.'

Method: immune serum competition

MSP-3$_{194-257}$ coated ELISA plates (Maxisorb, NUNC 475515, Denmark) was incubated with serial dilutions of either immune serum (from a malaria immune individual) or control serum. After two hours the plate was washed 5 times with PBS 0,05% Tween 20. Then FITC-conjugated RAM1 IgG1 antibody was applied in a dilution yielding maximum reactivity. FITC-conjugation was performed as described by the manufacturer (Molecular Probes, cat. No. F-6434). The plate was incubated for one hour, then it was washed 5 times and subsequently relative fluorescence was determined using a Polarstar fluorescence reader (BMG, Germany).

We conclude that the epitope recognized by RAM1 on the antigen is indeed one that is also recognized by the immune system of a malaria immune individual. This substantiates the clinical *in vivo* relevance of the RAM1 clone and thus also of clones RAM2 and RAM3 as the three clones bind to very closely related epitopes, see Figure 13.

Example 4   Production of eukaryotic IgG1 and IgG3

Methods

Plasmids

The eukaryotic expression of intact antibodies was performed with the two plasmids, pLNOH2 and pLNOK (Norderhaug et al. 1997).

The structure of these plasmids and their relation to the assembled antibody is shown in Figure 12 and Figure 14.

Briefly, the V-regions of the plasmids are exchanged by cutting with *Bsm*I in the 5´ end and by cutting with *Hpa*I or *Bsi*WI or *Hind*III in the 3´ end. Before ligation of vector and V region, the V regions of RAM1, RAM2 or RAM3 need to be cut with the same

restriction enzymes as the vectors. VH regions are then ligated to pLNOH2 and VK regions are ligated to pLNOK. The constant region of pLNOH2 can be exchanged by cutting with *BamHI* and *Hind*III, isolating the vector part and introducing (by ligation) another constant region cut with the same enzymes.

pLNOH2 is a vector for expression of heavy chain V-region genes in combination with any constant fragment (Fc) gene. Two versions of the vector were used, one with the human γ1 constant fragment gene and another with the human γ3 gene, to enable recombinant expression of the two isotypic variants of the antibody.

pLNOK is a vector for expression of kappa light chain V-region genes in combination with the kappa constant domain gene. The common features of the two vectors are briefly described below. The CMV promoter is situated 5' to a gene casette containing the leader region of murine immunoglobulin genes, the V-region gene inserted between restriction sites *BsmI* and *BsiWI* followed by an intron and the entire genomic C-region with a poly-A signal in the 3' end. The intron comprises splice acceptor and splice donor signals for correct processing of the mRNA. Non-abundant restriction sites flank the V-regions genes and are introduced by PCR into the gene fragments to be cloned. Additional features of the vectors are, ampicillin resistance marker for selection in *E. coli*, f1-origin of replication, Neomycin resistance selection marker for stable expression and the SV40 origin of replication to allow transient expression.

In order to produce a complete antibody, the plasmids (both pLNOH2 and pLNOK) are cotransfected into CHO cells. The V and C regions are assembled by mRNA splicing. When co-transfected in one cell, e.g. a CHO cell, the cell produces complete and correctly assembled antibodies.

As an alternative, the genes can be assembled in to one vector which thus harbours the VH, CH , VL and CL domains.

PCR of antibody genes

Two separate PCR amplifications were performed for each antibody, one for the heavy chain and one for the light chain. The templates were pFAB73H+RAM1, pFAB73H+RAM2 and pFAB73H+RAM3. For amplification of the RAM1 heavy chain V-region fragment one primer designated EuHVH-1 was based on the 5' sequence of RAM1 V_H (indicated in bold) and further contained the recognition sequence of restriction enzyme *Bsm*I (indicated by underlining and italics):

EuHVH-1:

5' ggt g*tg cat tcc* **cag gtn caa ttg gtr car tcy g** 3'

Ambiguous bases (highlighted by underlining) were introduced to make this primer compatible with the primer set published by Dziegiel et al. 1995.

The other primer, EuHJH, was complementary to the 3' sequence of RAM1 V_H (indicated in bold) and contained the additional sequence of the restriction enzymes *Hind*III, *Bsi*WI and *Hpa*I (indicated by underlining) and the complementary sequence to the splice donor sequence 5' AGGTGAGT 3' (indicated in capital letters):

EuHJH:

5' gt cca agc ttc gta cgt taA CTC ACC Tga rga gac rgt gac c 3'
         *Hind*III   *Bsi*WI   *Hpa*I

For amplification of the RAM1 light chain V-region gene fragment two primers designated EuHVKuniversal and EuHJK-14a were used. EuHVK universal was made compatible with the primer set published by Dziegiel et al. 1995 by taking advantage of the invariant 5' sequence of all kappa chain genes derived from this primer set (indicated by double underlining).

The primer further contained the recognition sequence of restriction enzyme *Bsm*I (indicated by bold) and part of the pelB

leader sequence found in the phage display vector (indicated by underlining):

EuHVKuniversal:

5' ttg tta tta ctc gcg gcc cag ccg gtg cat tcc gac atc 3'

|---------- PelB leader seq. -----|| BsmI | |-> Vκ

EuHJK-14a was based on the same principle as EuHJH shown above, complementarity to the 3' sequence of RAM1 V$_K$ (indicated in bold), the sequence of the restriction enzymes *Hind*III, *BsiW*I and *Hpa*I (indicated by underlining) and the sequence complementary to the splice donor sequence 5'A CTC ACG T 3' (indicated in capital letters):

EuHJK-14a:

g tcc aag ctt cgt acg tta acA CTC ACG Ttt gat ctc cag cct gg

For amplification of the RAM2 heavy chain V-region fragment one primer designated EuHVH-2 was based on the 5' sequence of RAM2 V$_H$ (indicated in bold) and further contained the recognition sequence of restriction enzyme *Bsm*I (indicated by underlining and italics):

EuHVH-2:

ggt *gtg cat tcc* cag gtg caa ttg cag gag tcs g

Ambiguous bases (highlighted by underlining) were introduced to make this primer compatible with the primer set published by Dziegiel et al. 1995.

The other primer, EuHJH is described above.

For amplification of the RAM2 light chain V-region gene fragment two primers designated EuHVKuniversal and EuHJK-15 were used. EuHVKuniversal is described above.

EuHJK-15 was based on the same principle as EuHJK-14a shown
above, complementarity to the 3' sequence of RAM2 V$_K$ (indicated in
bold), the sequence of the restriction enzymes *Hind*III, *BsiW*I and
*Hpa*I (indicated by underlining) and the sequence complementary to
the splice donor sequence 5'A CTC ACG T 3' (indicated in capital
letters):

EuHJK-15:

g tcc aag ctt cgt acg tta acA CTC ACG **Ttt gat ctc cac cc**

For amplification of the RAM3 heavy chain V-region fragment the
same primers were used as for RAM1 (see above).

For amplification of the RAM3 light chain V-region gene fragment
two primers designated EuHVKuniversal and EuHJK-8 were used.
EuHVKuniversal is described above.

EuHJK-8 was based on the same principle as EuHJK-14a shown above,
complementarity to the 3' sequence of RAM3 V$_K$ (indicated in bold),
the sequence of the restriction enzymes *Hind*III, *BsiW*I and *Hpa*I
(indicated by underlining) and the sequence complementary to the
splice donor sequence 5'A CTC ACG T 3' (indicated in capital
letters):

EuHJK-8:

gt cca agc ttc gta cgt taa ctt ctA CTC ACG **Ttt gat ytc cac ctt gg**

The PCR were performed using Gene Amp PCR System 9600, AmpliTaq
Gold and the supplied buffers. Primers were used in a final
concentration of 0,2 μM. The PCR cycling included the following
steps: 10 min. at 95°C (hot start, activation of AmpliTaq), 14
cycles of 94°C for 30 seconds, 40°C for 30 seconds and 72°C for 30
seconds and finally one cycle at 72°C for 10 min.

Amplification products of the expected sizes were isolated by gel
electrophoresis, purified with Gene Clean (Bio 101, Inc., CA,

USA) and ligated into pGem-T Easy (Promega, USA) according to the manufacturer.

Ligations were used to transform electrocompetent XL-1 blue (Stratagene). Subsequently the cells were plated on LB containing X-gal and IPTG as described in Sambrook et al. 1989. White clones were sequenced using Big dye (Applied Biosystems, USA) and the primers SP-6 and T7 as recommended by the manufacturer. The sequence reactions were applied to ABI PRISM Sequencer 310 (Perkin Elmer, USA).

For each heavy and light chain a clone was selected that was either identical to the template or that did not alter the deduced amino acid sequence.

The selected clones were digested with *BsmI* and *BsiWI* or with *BsmI* and *HindIII*. The fragments were then ligated into the appropriate vector digested with the same enzymes.

Cell line

CHO cells were used as eukaryotic host cells for stable expression of intact antibodies.

Transfection and expression

Cells were grown to 50-80% confluency in Ham's F-12 Medium supplemented with 10% inactivated Foetal Bovine Serum (Life Technologies). Cells were then transfected with Lipofectamine (Life Technologies) according to the procedures of the manufacturers. The following day transfected cells were transferred to microtiter plates to select clones by limiting dilution. After approximately 14 days wells containing single cell colonies were tested for production of anti-MSP-3 antibodies in ELISA. Cells from approx. 20 wells giving the highest response in the ELISA assay were transferred to new wells in a 24 well microtiter plate, grown to confluence, transferred to new wells in a 6 well microtiter plate, grown to confluence and finally transferred to two 25 cm^2 TC flasks. Cells in one flask

were grown to confluence and sebsequently frozen. Cells in the
second flask were grown for approx. 4 weeks whereupon the
supernatant was tested for production of anti-MSP-3 antibodies in
ELISA. The clone giving the highest response in the ELISA assay
was used for a second round of limited dilution. The clone from
the second round of limited dilution giving the highest response
in the ELISA assay was grown to an appropriate number of cell to
be used for 1) freezing and 2) expression of antibody. For
expression cells were plated to approx. 10% confluence in triple
TC flasks (NUNC, Denmark) and grown for approx. 4 weeks. The
supernatant was centrifuged 20,000 x g for 30 minutes before use
in subsequent assays to remove cellular debris.

Immunochemistry

The composition of human antibodies in the supernatant from the
cell culture was examined by capture ELISA. Human antibodies
were captured on a coating of goat anti-human Fab fragments
(Sigma I5260) and detected with mouse anti-human IgG1 (Zymed 05-
3600) or IgG3 (Zymed 05-3300), respectively, followed by rabbit
anti-mouse conjugated to horse radish peroxidase (DAKO P260).
ELISA for reactivity with specific antigen was performed as
described under Example 3. Furthermore, the IgG1 version of
RAM1 and RAM2 were used for Western blotting and for flow
cytometry.

Capture ELISA for quantitation of IgG

Capturing of recombinant IgG was done with goat anti-human IgG
specific for the Fc-portion 1:5,000 (Sigma I2136), detection was
performed with alkaline phosphatase conjugated goat anti-human
kappa chain 1:10,000 (Sigma A3813). Purified human recombinant
anti-D IgG1, kappa (751 µg/mL) and IgG3, kappa (135 µg/mL),
respectively, were used as standards. ELISA procedures and
buffers were as described in Dziegiel et al. 1991.

Results

The V$_H$ and V$_K$ genes derived from the anti-MSP-3 antibodies were combined with constant domain genes in plasmids pLNOH2 and pLNOK, respectively. Two different versions of the heavy chain vector, pLNOH2, were used, one version containing the γ1 constant domain gene and the other containing the γ3 constant domain gene. These two vectors were used for production of an IgG1 version as well as an IgG3 version of the anti-MSP-3 antibodies. The composition of the antibodies was investigated by immunochemistry in ELISA. The specificity of the IgG1 and IgG3 versions was tested with ELISA technique.

Example 6   Purification of Eukaryotic IgG1 and IgG3

After ammonium sulfate precipitation (70%), pelleted proteins were resuspended in water. Buffer exchange to 70 mM Sodium acetate pH 5.0 was performed by dialysis. Recombinant IgG was purified using DEAE Sepharose FF (Pharmacia BioTech) followed by applying the flow through to an ABx column (Baker Bond). The buffer was changed to 50 mM Bicine pH 8.5 and the IgG eluted with a gradient of 50 mM Bicine supplemented with 0.5 M NaCl pH 8.5. If necessary antibody containing fractions from the Abx column are precipitated by ammonium sulphate as above and applied to the gel filtration column (Superdex200). The purity was checked by gel chromatography using Superdex200 and by SDS-PAGE followed by silver staining.

Example 7   Characterization of the biological activity of recombinant antibodies by ADCI and other functional and parasitological tests

These assays reflect the clinical status of protection. The ADCI reflects the capacity of individual IgG to inhibit *P. falciparum* growth *in vitro* by cooperation with human blood monocytes as described in Bouharoun-Tayounet al. 1995. The *P. falciparum* merozoite phagocytosis assay in the presence of antibody has been checked among the 200 inhabitants from Dielmo where it closely reflects the clinical status of the inhabitants.

ADCI and phagocytosis assays are performed with the selected antibodies and human monocytes following standardized procedures.

5   For antibodies expressed with Fc portions in eukaryotic cells, the classical ADCI assay is used, as described by Shi et al. 1999.

The parasites are cultured in the presence of recombinant
10   antibodies and healthy donor derived monocytes. In order to determine the specific growth inhibition (SGI) of ADCI described below, the appropriate controls are included.

$$SGI\ of\ ADCI = 100x \left(1 - \frac{\left(\dfrac{\%\ mean\ parasitemia\ with\ test\ IgG\ and\ monocytes}{\%\ mean\ parasitemia\ with\ test\ IgG} \right)}{\left(\dfrac{\%\ mean\ parasitemia\ with\ control\ IgG\ and\ monocytes}{\%\ mean\ parasitemia\ with\ control\ IgG} \right)} \right)$$

15
Monocytes are isolated by the lymfoprep method, and by adhesion to autologous plasma coated plastic tissue culture Petri dishes (Nunc).

20   The malaria parasite is grown as described by Trager and Jensen 1976 in 96 well tissue culture plates. The effect of IgG's and monocytes is determined in 48-hour cultures with an initial parasitemia of 0,5%. For tests with monocytes these are activated by rhIFN-γ and added to each well.

25
After 48 hours the parasitemias are assessed by flowcytometry as described by Heyde et al. 1995. The cultures are after 48 hours incubated with hydroethidine which is a vital dye that is converted to ethidium by metabolically active cells. Ethidium
30   interacts with the parasite derived DNA in infected cells and thereby allows discrimination between infected and non-infected erythrocytes by flowcytometry (Shi et al. 1999).

A flowcytometry based phagocytosis assay developed by
35   Kumaratilake and Ferrante (2000) will be used to determine the

opsonization and phagocytosis mediating abilities of the
produced antibodies. This assay explores the ability of
neutrophils to quench FITC-conjugated merozoites. This assay is
adapted to detect phagocytosis by other leukocytes (such as
monocytes) as an effect of antibody opsonization (Kushmith &
Druilhe, 1983.

Furthermore, the antibodies produced are tested *in vivo* in the
mouse model developed by Badell et al (1995) & (2000). This
model is based on the possibility of obtaining a *sustained P.
falciparum* growth in immunocompromised mice reconstituted with
human erythrocytes as well as human monocytes.

DISCUSSION

The strategy followed for construction of the library was to
attempt to clone as many antibody genes as possible from the 13
malaria immune individuals from Senegal, Africa. This presents
some important advantages as opposed to the strategy of trying
to cover the entire repertoire from only a single or a few
individuals. Immunity to malaria is supposed to be one of the
major selective pressures exerted to mankind in malaria endemic
areas during evolution. It has been postulated (Antibody
repertoires and pathogen recognition: the role of germline
diversity and somatic hypermutation. PhD thesis by Mihaela
Oprea, 1999, Santa Fe Institute) that an important factor in
successful development of malaria immunity in an individual is
the possession of a limited number of selected heavy chain
genes, e.g. 10^5, encoding broadly reactive antibodies. The role
of such antibodies would be to interact with all important and
harmful antigens in order to initiate an antibody response
progressively leading to a higher quality response in one and
the same individual. The higher quality is manifested in
specificity, affinity and protective biological effects. By
cloning $5x10^7$ different heavy chain genes from 13 individuals
the library on the average contains $4x10^6$ different genes from
each individual thus enhancing the likelihood of having cloned
at least one copy of each of the 10^5 important genes. The other
strategy, relying on cloning a similar size library from one

individual would at the most result in the cloning of one full repertoire of the 10^5 basic repertoire of antibodies.

The antibodies provided here provide a novel strategy for
5   prevention and treatment of malaria.   If ordinary ADCC and phagocytosis were the sole mechanisms at play a high initial parasitemia would lead to a large consumption of immunoglobulin and consequently to a lower fractional reduction in parasitemia. Therefore it is believed that the antibody-merozoite complex
10   stimulates the monocyte in a contact-dependent manner to secrete the effector substance(s) responsible for a subsequent non-contact-dependent inhibition of the growth of the parasite. Candidate molecules for the inhibitory substances are nitrogen oxide and TNF-α.   Thus the high initial concentration of
15   parasites is assumed to induce a high and efficient concentration of anti-parasitic substances leading to a high reduction rate.

Exploitation of the naturally occurring anti-parasitic
20   host/pathogen interaction (premunition, ADCI) is a novel way of developing a therapeutical anti-parasitic activity.   The immunological mechanism that is exploited by these antibodies is the co-operation between the three components, merozoite - antibody - monocyte leading to inhibition of parasite growth
25   (ADCI) through secretion of TNF-α and other substances, e.g. nitrogen oxide species and oxygen radicals.   This mechanism of protection requires only a minority of the merozoites to interact physically with antibody and monocyte.   Indeed, the majority of the parasites are only exposed to the substances
30   released as a consequence of the merozoite-antibody-monocyte interaction.

The use of recombinant antibodies ensures that the appropriate isotype can be used.   This would not be the case if antibodies
35   purified from infectious individuals were used, e.g. for the ADCI mechanism, IgG1 and IgG3 isotypes are important.

The sub-populations of target antigen-escape mutants will not have a direct survival advantage compared to the wild-type and

therefore do not influence the general level of anti-parasitic
activity. Only in the situation where the number of wild-type
parasites had been reduced dramatically would the monocyte
stimulus drop. A minority of parasites possessing wild-type
5   target-antigen would be sufficient for stimulating the monocyte
to release substances with anti-parasitic activity. Therefore,
resistance to this anti-parasitic mechanism is a minor concern.
Studies of the conservation of the relevant domains of the
target molecule MSP-3, have indeed confirmed that immunological
10   selection pressure seemingly is absent as a consequence of the
above-mentioned immunological mechanism (Stricker et al 1999).

Insensitivity of the parasites to the action of the substances
released by monocytes and other effector cells does not seem to
15   be a problem. This is substantiated by the clinical observation
of life-long persistence of the so-called premunition once it
has been obtained and is maintained by continuous exposure to
the parasite.

20   Resistance to this mechanism will be a minor concern because it
mimics and exploits indirect control mechanisms established by
host/pathogen co-existence through thousands and thousands of
generations. Therefore, these antibodies provide an anti-
parasitic effect without the risk of induction of parasite
25   resistance.

Use of recombinant antibodies circumvents the uncertainty
connected with the parameters influencing each step in the
classical vaccine development process: Antigen, vaccine
30   formulation, immunogenicity, individual immune response genes,
immune response and finally, the interaction of
host/parasite/antibody.

REFERENCES

1. Aribot G., Rogier C., Sarthou J.L., Trape J.F., Balde
A.T., Druilhe P. and Roussilhon C. (1996) Pattern of
immunoglobulin isotype response to Plasmodium falciparum
blood-stage antigens in individuals living in a
holoendemic area of Senegal (Dielmo, west Africa).
Am.J.Trop.Med.Hyg. **54**, 449-457.

2. Ausubel F.M. (1998) *Current protocols in molecular
biology.* John Wiley & Sons, Inc.

3. Badell E., Oeuvray C., Moreno A., Soe S., van Rooijen N.,
Bouzidi A. and Druilhe P. (2000) Human malaria in
immunocompromised mice: an in vivo model to study defense
mechanisms against Plasmodium falciparum. *J.Exp.Med.* **192**,
1653-1660.

4. Bass S., Greene R. and Wells J.A. (1990) Hormone phage:
an enrichment method for variant proteins with altered
binding properties. *Proteins* **8**, 309-314.

5. Bird R.E., Hardman K.D., Jacobson J.W., Johnson S.,
Kaufman B.M., Lee S.M., Lee T., Pope S.H., Riordan G.S.
and Whitlow M. (1988) Single-chain antigen-binding
proteins. *Science* **242**, 423-426.

6. Bouharoun-Tayoun H., Attanath P., Sabchareon A.,
Chongsuphajaisiddhi T. and Druilhe P. (1990) Antibodies
that protect humans against Plasmodium falciparum blood
stages do not on their own inhibit parasite growth and
invasion in vitro, but act in cooperation with monocytes.
J.Exp.Med. **172**, 1633-1641.

7. Bouharoun-Tayoun H. and Druilhe P. (1992) Plasmodium
falciparum malaria: evidence for an isotype imbalance
which may be responsible for delayed acquisition of
protective immunity. *Infect.Immun.* **60**, 1473-1481.

8.   Bouharoun-Tayoun H., Oeuvray C., Lunel F. and Druilhe P.
      (1995) Mechanisms underlying the monocyte-mediated
      antibody-dependent killing of Plasmodium falciparum
      asexual blood stages. *J.Exp.Med.* **182**, 409-418.

9.   Chirgwin J.M., Przybyla A.E., MacDonald R.J. and Rutter
      W.J. (1979) Isolation of biologically active ribonucleic
      acid from sources enriched in ribonuclease. *Biochemistry*
      18, 5294-5299.

10.   Clackson T., Hoogenboom H.R., Griffiths A.D. and Winter
      G. (1991) Making antibody fragments using phage display
      libraries. *Nature* **352**, 624-628.

11.   Crameri A., Cwirla S. and Stemmer W.P. (1996)
      Construction and evolution of antibody-phage libraries by
      DNA shuffling. *Nat.Med.* **2**, 100-102.

12.   Druilhe P. and Khusmith S. (1987) Epidemiological
      correlation between levels of antibodies promoting
      merozoite phagocytosis of Plasmodium falciparum and
      malaria-immune status. *Infect.Immun.* **55**, 888-891.

13.   Dziegiel M., Borre M.B., Jepsen S., Hogh B., Petersen E.
      and Vuust J. (1991) Recombinant Plasmodium falciparum
      glutamate rich protein; purification and use in enzyme-
      linked immunosorbent assay. *Am.J.Trop.Med.Hyg.* **44**, 306-
      313.

14.   Dziegiel M., Nielsen L.K., Andersen P.S., Blancher A.,
      Dickmeiss E. and Engberg J. (1995) Phage display used for
      gene cloning of human recombinant antibody against the
      erythrocyte surface antigen, rhesus D. *J.Immunol.Methods*
      182, 7-19.

15.   Engberg J., Andersen P.S., Nielsen L.K., Dziegiel M.,
      Johansen L.K. and Albrechtsen B. (1996) Phage-display

libraries of murine and human antibody Fab fragments.
Mol.Biotechnol. **6,** 287–310.

16.   Holliger P., Prospero T. and Winter G. (1993)
      "Diabodies": small bivalent and bispecific antibody
      fragments. *Proc.Natl.Acad.Sci.U.S.A* **90,** 6444–6448.

17.   Hoogenboom H.R., Griffiths A.D., Johnson K.S., Chiswell
      D.J., Hudson P. and Winter G. (1991) Multi-subunit
      proteins on the surface of filamentous phage:
      methodologies for displaying antibody (Fab) heavy and
      light chains. *Nucleic Acids Res.* **19,** 4133–4137.

18.   Hu S., Shively L., Raubitschek A., Sherman M., Williams
      L.E., Wong J.Y., Shively J.E. and Wu A.M. (1996)
      Minibody: A novel engineered anti-carcinoembryonic
      antigen antibody fragment (single-chain Fv-CH3) which
      exhibits rapid, high-level targeting of xenografts.
      Cancer Res. **56,** 3055–3061.

19.   Huber W., Felger I., Matile H., Lipps H.J., Steiger S.
      and Beck H.P. (1997) Limited sequence polymorphism in the
      Plasmodium falciparum merozoite surface protein 3.
      Mol.Biochem.Parasitol. **87,** 231–234.

20.   Huston J.S., Levinson D., Mudgett-Hunter M., Tai M.S.,
      Novotny J., Margolies M.N., Ridge R.J., Bruccoleri R.E.,
      Haber E., Crea R. and . (1988) Protein engineering of
      antibody binding sites: recovery of specific activity in
      an anti-digoxin single-chain Fv analogue produced in
      Escherichia coli. *Proc.Natl.Acad.Sci.U.S.A* **85,** 5879–5883.

21.   Jirholt P, Ohlin M, Borrebaeck CA, Soderlind E. (1998).
      Exploiting sequence space: shuffling in vivo formed
      complementarity determining regions into a master
      framework.   Gene 1998 Jul 30; 215(2):417–6

22.   Kabat E.A. and National Institutes of Health ((1991)
      Sequences of proteins of immunological interest. U.S.

Dept. of Health and Human Services, Public Health
Service, National Institutes of Health, Bethesda, Md.

23. Khusmith S. and Druilhe P. (1983) Antibody-dependent
    ingestion of P. falciparum merozoites by human blood
    monocytes. *Parasite Immunol.* **5**, 357-368.

24. Kumaratilake L.M. and Ferrante A. (2000) Opsonization and
    phagocytosis of Plasmodium falciparum merozoites measured
    by flow cytometry. *Clin.Diagn.Lab Immunol.* **7**, 9-13.

25. Marks J.D., Griffiths A.D., Malmqvist M., Clackson T.P.,
    Bye J.M. and Winter G. (1992) By-passing immunization:
    building high affinity human antibodies by chain
    shuffling. *Biotechnology (N.Y.)* **10**, 779-783.

26. Marks J.D., Hoogenboom H.R., Bonnert T.P., McCafferty J.,
    Griffiths A.D. and Winter G. (1991) By-passing
    immunization. Human antibodies from V-gene libraries
    displayed on phage. *J.Mol.Biol.* **222**, 581-597.

27. McColl D.J. and Anders R.F. (1997) Conservation of
    structural motifs and antigenic diversity in the
    Plasmodium falciparum merozoite surface protein-3 (MSP-
    3). *Mol.Biochem.Parasitol.* **90**, 21-31.

28. McColl D.J., Silva A., Foley M., Kun J.F., Favaloro J.M.,
    Thompson J.K., Marshall V.M., Coppel R.L., Kemp D.J. and
    Anders R.F. (1994) Molecular variation in a novel
    polymorphic antigen associated with Plasmodium falciparum
    merozoites. *Mol.Biochem.Parasitol.* **68**, 53-67.

29. McGregor I.A. (1964) The passive transfer of human
    malarial immunity. *Am.J.Trop.Med.Hyg.* **13**, 237-239.

30. McGregor I.A., Carrington S. and Cohen S. (1963)
    Treatment of East African P. falciparum malaria with West
    African human gamma-globulin. *Trans.R.Soc.Trop.Med.Hyg.*
    57, 170-175.

31. Mulhern T.D., Howlett G.J., Reid G.E., Simpson R.J., McColl D.J., Anders R.F. and Norton R.S. (1995) Solution structure of a polypeptide containing four heptad repeat units from a merozoite surface antigen of Plasmodium falciparum. *Biochemistry* **34**, 3479-3491.

32. Norderhaug L., Olafsen T., Michaelsen T.E. and Sandlie I. (1997) Versatile vectors for transient and stable expression of recombinant antibody molecules in mammalian cells. *J.Immunol.Methods* **204**, 77-87.

33. Oeuvray C., Bouharoun-Tayoun H., Gras-Masse H., Bottius E., Kaidoh T., Aikawa M., Filgueira M.C., Tartar A. and Druilhe P. (1994) Merozoite surface protein-3: a malaria protein inducing antibodies that promote Plasmodium falciparum killing by cooperation with blood monocytes. *Blood* **84**, 1594-1602.

34. Oeuvray C., Theisen M., Rogier C., Trape J.F., Jepsen S. and Druilhe P. (2000) Cytophilic immunoglobulin responses to Plasmodium falciparum glutamate- rich protein are correlated with protection against clinical malaria in Dielmo, Senegal. *Infect.Immun.* **68**, 2617-2620.

35. Ohlin M., Owman H., Mach M. and Borrebaeck C.A. (1996) Light chain shuffling of a high affinity antibody results in a drift in epitope recognition. *Mol.Immunol.* **33**, 47-56.

36. Oprea, M. L. Antibody repertoires and pathogen recognition: The role of germline diversity and somatic hypermutation. 1-5-1999. The University of New Mexico. Ref Type: Thesis/Dissertation

37. Orum H., Andersen P.S., Oster A., Johansen L.K., Riise E., Bjornvad M., Svendsen I. and Engberg J. (1993) Efficient method for constructing comprehensive murine

Fab antibody libraries displayed on phage. *Nucleic Acids Res.* **21**, 4491-4498.

38. Persic L., Roberts A., Wilton J., Cattaneo A., Bradbury A. and Hoogenboom H.R. (1997) An integrated vector system for the eukaryotic expression of antibodies or their fragments after selection from phage display libraries. *Gene* **187**, 9-18.

39. Reiter Y., Brinkmann U., Lee B. and Pastan I. (1996) Engineering antibody Fv fragments for cancer detection and therapy: disulfide-stabilized Fv fragments. *Nat.Biotechnol.* **14**, 1239-1245.

40. Sambrook J., Maniatis T. and Fritsch E.F. (1989) *Molecular cloning a laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

41. Sanna P.P., Samson M.E., Moon J.S., Rozenshteyn R., De Logu A., Williamson R.A. and Burton D.R. (1999) pFab-CMV, a single vector system for the rapid conversion of recombinant Fabs into whole IgG1 antibodies. *Immunotechnology.* **4**, 185-188.

42. Seehaus T., Breitling F., Dubel S., Klewinghaus I. and Little M. (1992) A vector for the removal of deletion mutants from antibody libraries. *Gene* **114**, 235-237.

43. Sheeley D.M., Merrill B.M. and Taylor L.C. (1997) Characterization of monoclonal antibody glycosylation: comparison of expression systems and identification of terminal alpha-linked galactose. *Anal.Biochem.* **247**, 102-110.

44. Shi Y.P., Udhayakumar V., Oloo A.J., Nahlen B.L. and Lal A.A. (1999) Differential effect and interaction of monocytes, hyperimmune sera, and immunoglobulin G on the growth of asexual stage Plasmodium falciparum parasites. *Am.J.Trop.Med.Hyg.* **60**, 135-141.

52

45.   Trager W. and Jensen J.B. (1976) Human malaria parasites
        in continuous culture. *Science* **193**, 673-675.

46.   Trucco C., Fernandez-Reyes D., Howell S., Stafford W.H.,
        Scott-Finnigan T.J., Grainger M., Ogun S.A., Taylor W.R.
        and Holder A.A. (2001) The merozoite surface protein 6
        gene codes for a 36 kDa protein associated with the
        Plasmodium falciparum merozoite surface protein-1
        complex. *Mol.Biochem.Parasitol.* **112**, 91-101.

47.   van der Heyde H.C., Elloso M.M., vande W.J., Schell K.
        and Weidanz W.P. (1995) Use of hydroethidine and flow
        cytometry to assess the effects of leukocytes on the
        malarial parasite Plasmodium falciparum. *Clin.Diagn.Lab
        Immunol.* **2**, 417-425.

48.   Walls M.A., Hsiao K.C. and Harris L.J. (1993) Vectors for
        the expression of PCR-amplified immunoglobulin variable
        domains with human constant regions. *Nucleic Acids Res.*
        21, 2921-2929.

49.   Ward E.S., Gussow D., Griffiths A.D., Jones P.T. and
        Winter G. (1989) Binding activities of a repertoire of
        single immunoglobulin variable domains secreted from
        Escherichia coli. *Nature* **341**, 544-546.

CLAIMS:

1.   A recombinant human antibody comprising an antibody
sequence specific for the MSP-3 antigen of *Plasmodium*
falciparum.

2.   A recombinant human antibody according to claim 1 which
is specific for the MSP-$3_{194-257}$ antigen.

3.   A recombinant human antibody according to claim 2 which
is specific for an epitope having the amino acid sequence
ILGWEFGGGVP which corresponds to residues 220-230 of the MSP-3
antigen.

4.   A recombinant human antibody according to any one of the
preceding claims which comprises a CDR sequence selected from:
CDR1 of RAM1 VH; CDR2 of RAM1 VH; CDR3 of RAM1 VH; CDR1 of RAM1
VK; CDR2 of RAM1 VK; CDR3 of RAM1 VK; CDR1 of RAM2 VH; CDR2 of
RAM2 VH; CDR3 of RAM2 VH; CDR1 of RAM2 VK; CDR2 of RAM2 VK; CDR3
of RAM2 VK; CDR1 of RAM3 VH; CDR2 of RAM3 VH; CDR3 of RAM3 VH;
CDR1 of RAM3 VK; CDR2 of RAM3 VK; or CDR3 of RAM3 VK having the
amino acid sequences shown underlined in Figure 6.

5.   A recombinant human antibody according to claim 4
comprising a CDR selected from: CDR3 of RAM1 VH; CDR3 of RAM2
VH; or CDR3 of RAM3 VH having the sequence as shown in Figure 6.

6.   A recombinant human antibody according to claim 4 or
claim 5 comprising two or more CDRs selected from: CDR1 of RAM1
VH; CDR2 of RAM1 VH; CDR3 of RAM1 VH; CDR1 of RAM1 VK; CDR2 of
RAM1 VK; CDR3 of RAM1 VK; CDR1 of RAM2 VH; CDR2 of RAM2 VH; CDR3
of RAM2 VH; CDR1 of RAM2 VK; CDR2 of RAM2 VK; CDR3 of RAM2 VK;
CDR1 of RAM3 VH; CDR2 of RAM3 VH; CDR3 of RAM3 VH; CDR1 of RAM3
VK; CDR2 of RAM3 VK; or CDR3 of RAM3 VK having the sequence
shown in Figure 6.

7.   A recombinant human antibody according to claim 6 wherein
the two or more CDRs are from the same RAM clone.

8.   A recombinant human antibody according to claim 7 wherein
CDR1, CDR2, CDR3 of the light chain and CDR1, CDR2 and CDR3 of
the heavy chain are all from the same RAM clone.

9.   A recombinant human antibody according to claim 8 wherein
the CDRs are all from RAM1.

10.   A recombinant human antibody according to claim 8 wherein
the CDRs are all from RAM2.

11.   A recombinant human antibody according to claim 8 wherein
the CDRs are all from RAM3.

12.   A recombinant human antibody according to claim 6
comprising the V_H domain of RAM1 having the amino acid sequence
shown as RAM1 VH in Figure 6 (the entire top line of the first
box in Figure 6).

13.   A recombinant human antibody according to claim 6
comprising the V_H domain of RAM2 having the amino acid sequence
shown as RAM2 VH in Figure 6 (the entire top line of the second
box in Figure 6).

14.   A recombinant human antibody according to claim 6
comprising the V_H domain of RAM3 having the amino acid sequence
shown as RAM3 VH in Figure 6 (the entire top line of the third
box in Figure 6).

15.   A recombinant human antibody according to claim 6
comprising the V_L domain of RAM1 having the amino acid sequence
shown as RAM1 VK in Figure 6 (the entire bottom line of the
first box in Figure 6).

16.   A recombinant human antibody according to claim 6
comprising the V_L domain of RAM2 having the amino acid sequence
shown as RAM2 VK in Figure 6 (the entire bottom line of the
second box in Figure 6).

17. A recombinant human antibody according to claim 6 comprising the V_L domain of RAM3 having the amino acid sequence shown as RAM3 VK in Figure 6 (the entire bottom line of the third box in Figure 6).

18. A recombinant human antibody according to claim 12 further comprising the V_L domain of RAM1 having the amino acid sequence shown as RAM1 VK in Figure 6 (the entire bottom line of the first box in Figure 6).

19. A recombinant human antibody according to claim 13 further comprising the V_L domain of RAM2 having the amino acid sequence shown as RAM2 VK in Figure 6 (the entire bottom line of the second box in Figure 6).

20. A recombinant human antibody according to claim 14 further comprising the V_L domain of RAM3 having the amino acid sequence shown as RAM3 VK in Figure 6 (the entire bottom line of the third box in Figure 6).

21. A recombinant human antibody according to any one of the preceding claims which comprises a constant region selected from: gamma 1; gamma 2; gamma 3; gamma 4; my; alfa 1; alfa 2; delta; or epsilon isotypes.

22. A recombinant human antibody according to claim 21 wherein the constant region is selected from: the gamma 1; gamma 2; gamma 3; or gamma 4 isotypes, to form an IgG molecule.

23. A recombinant human antibody according to claim 22 wherein the constant region is selected from: the gamma 1 or the gamma 3 isotypes, to form an IgG1 or IgG3 isotype.

24. An isolated nucleic acid molecule comprising a nucleic acid sequence encoding a recombinant human antibody according to any one of the preceding claims.

25. A nucleic acid molecule according to claim 24 in the form of a recombinant vector.

26. A host cell comprising a nucleic acid according to claim 25.

27. A method of expressing in a host cell an antibody according to any one of the preceding claims from a nucleic acid molecule according to claim 25.

28. A method of producing an antibody comprising culturing a host cell according to claim 26 under appropriate conditions.

29. A method according to claim 28 further comprising isolating said antibody from the cell culture.

30. A method according to claim 29 further comprising admixing the isolated antibody with a suitable further component, such as another antibody or an excipient or carrier.

31. A recombinant human antibody according to any one of claims 1 to 23 for use in the treatment of a malarial disease.

32. The use of a recombinant human antibody according to any one of claims 1 to 23 in the manufacture of a medicament for the treatment malaria.

33. A recombinant human antibody according to claim 31 or use according to claim 32 wherein the dose is 1 mg to 5 mg antibody per kg body weight.

34. A recombinant human antibody or use according to claim 33 wherein the dose is 1.5 mg to 3.5 mg antibody per kg body weight.

35. A recombinant human antibody or use according to claim 34 wherein the dose is 2 mg antibody per kg body weight.

36. A recombinant human antibody according to any one of claims 1 to 23 for use in the prophylaxis of a malarial disease.

37.   The use of a recombinant human antibody according to any one of claims 1 to 23 in the manufacture of a medicament for the prophylaxis of a malarial disease.

38.   A recombinant human antibody according to claim 36 or use according to claim 37 wherein the dose is 0.1 mg to to 3 mg antibody per kg body weight.

39.   A recombinant human antibody or use according to claim 38 wherein the dose is 0.2 mg to 2 mg antibody per kg body weight.

40.   A recombinant human antibody or use according to claim 39 wherein the dose is 0.5 mg to 1.5 mg antibody per kg body weight.

41.   A recombinant human antibody according to claim 40 wherein the dose is 0.75 mg to 1 mg antibody per kg body weight.

42.   A recombinant human antibody or use according to claim 41 wherein the dose is most preferably 1 mg antibody per kg body weight.

43.   A nucleic acid molecule according to claim 24 or 25 for use in the treatment or prophylaxis of a malarial disease.

44.   Use of a nucleic acid molecule according to claim 24 or 25 in the treatment or prophylaxis of a malarial disease.

45.   A method comprising causing or allowing binding of an antibody according to any one of claims 1 to 23 to a target antigen.

46.   A method of diagnosis of malaria comprising taking a sample of bodily fluid from an individual, contacting the sample with an antibody according to any one of claims 1 to 23 and determining the binding of that antibody to the sample, thereby determining the presence or absence of a target antigen in the sample.

47. A method for screening a phage display library comprising the following steps:

(i) attaching a target molecule (such as an antigen) to a bead using a suitable linker;

(ii) admixing a first amount of such beads with attached target molecule with a first population of phage from a phage display library (which library expresses sequences (such as antibody sequences) which may bind to the target molecule;

(iii) selecting from the first population of phage a second population of phage, wherein the second population of phage is enriched in phage which bind to the target molecule;

(iv) admixing said second population of phage with a second amount of beads, wherein said second amount of beads is smaller than said first amount;

(v) selecting from said second population of phage a third population of phage, wherein said third population of phage enriched in phage which bind to the target molecule.

48. A method according to claim 47 wherein steps (iv) and (v) are repeated to produce a fourth population of phage.

49. A method according to claim 47 or claim 48 wherein the target molecule is an antigen and the phage display libaray contains antibody sequences.

50. A method according to any one of claims 47 to 49 further comprising the step of producing a complete antibody.

51. An antibody produced or producible by the method of claim 50.

Application No: GB 0120057.5   5ᑫ   **Examiner:**   Dr J Houlihan
Claims searched: 1-43, 45 & 46   **Date of search:**   4 October 2001

Patents Act 1977
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Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

    UK Cl (Ed.S):

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Other: ONLINE: WPI, EPODOC, PAJ, BIOSIS

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
A	WO 01/36587 A (HUMAN-BIO CO. LTD) whole document	
A	US 4897354 (Siddiqui W A) whole document	
Y	J. Biol. Chem. Vol. 276 (22) 2001. Roeffen W F G *et.al. "Recombinant human antibodies specific for the Pfs48/45 protein of the malaria parasite Plasmodium falciparum"* pages 19807-19811	1, at least
Y	Blood Vol 84 (5) 1994. Oeuvray C *et.al. " Merozoite surface protein-3: a malaria protein inducing antibodies that promote plasmodium falciparum killing by cooperation with blood monocytes"* pages1594-1602	1, at least
Y	Immunotechnology Vol. 2 (4) 1996. Dziegiel M et.al. *"Recombinant human antibodies against the Plasmodium falciparum glutamate rich protein"* page 299	1, at least
Y	J. Cellular Biol. (Suppl) 18D 1994. Sylvester D R *et.al. "Construction and in-vitro characterization of a humanised monoclonal antibody against Plasmodium falciparum sporozoites"*	1, at least

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.